



GONADOTROPIN-RELEASING
HORMONE:
MOLECULES AND
RECEPTORS

VOLUME 141

ISHWAR S. PARHAR

PROGRESS IN BRAIN RESEARCH

VOLUME 141

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AMSTERDAM – BOSTON – LONDON – NEW YORK – OXFORD – PARIS
SAN DIEGO – SAN FRANCISCO – SINGAPORE – SYDNEY – TOKYO

2002

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First edition 2002

Library of Congress Cataloging-in-Publication Data

Gonadotropin-releasing hormone : molecules and receptors / edited by Ishwar S.

Parhar.-- 1st ed.

p. ; cm. -- (Progress in brain research ; v. 141)

Includes bibliographical references and index.

ISBN 0-444-50979-8 (alk. paper) -- ISBN 0-444-80104-9 (series)

1. Luteinizing hormone releasing hormone. 2. Luteinizing hormone releasing hormone--Receptors. 3. Neurochemistry. I. Parhar, Ishwar S., 1958- II. Series.

[DNLN: 1. Gonadotropins--physiology. 2. Receptors, Gonadotropin--physiology. WK 900 G6378 2002]

QP572.L85 G655 2002

573.6'374--dc21

2002034691

British Library Cataloguing in Publication Data

A catalogue record from the British Library has been applied for.

ISBN: 0-444-50979-8 (volume)

ISBN: 0-444-80104-9 (series)

ISSN: 0079-6123

⊗ The paper used in this publication meets the requirements of ANSI/NISO Z39.48-1992 (Permanence of Paper).
Printed in The Netherlands.

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Preface

Gonadotropin-releasing hormone (GnRH, previously called luteinizing-hormone releasing hormone = LHRH) represents the first step in a cascade of events coordinating the complex physiology of reproduction and reproductive behavior in vertebrates. It was initially assumed that GnRH occurred as a single molecular form, in the septo-preoptic area of the vertebrate brain, and that it served a single function. However, over the last decade, 13 structurally distinct forms of GnRHs have been isolated from different vertebrate species. Recent research has also demonstrated the existence of GnRH receptor subtypes, which have evolved in conjunction with distinct GnRH ligands. The multiplicity of GnRH molecules and receptors has recently expanded with the discovery of chicken II GnRH in the brain of humans and other mammals. It has become increasingly clear that, all vertebrate species investigated to date possess two or three different GnRH genes, and that GnRH has multiple functions in addition to stimulating the release of gonadotropins. Non-mammalian vertebrate species have played a central role in the discovery of molecular diversity of GnRHs. Furthermore, comparative studies using different vertebrate models show remarkable similarity in the distribution pattern of GnRH neurons. The embryonic origin of GnRH neurons from the olfactory placode and their subsequent migration into the septo-preoptic area is unique among brain cells, which is evolutionarily conserved among vertebrate species ranging from fish to humans.

This volume summarizes the evolution and physiology of GnRH molecules and receptors, and provides insight as to how social behavior influences cellular and molecular events in the brain from a comparative perspective. The chapters in this volume are divided into three major sections: Development and Cell Migration, GnRH Receptors, and Physiology and Regulation. The review papers arose primarily from presentations made at the Second International Symposium on the Comparative Biology of GnRH, held in Penang, Malaysia, June 2–4, 2001; a satellite symposium in conjunction with the XIV International Congress of Comparative Endocrinology, Sorrento, Italy. In addition, leading neuroscientists doing cutting-edge research in the field of GnRH were invited as authors to make this volume a valuable reference.

I would like to acknowledge the cooperation and support received from the authors. I am indebted to those who helped in organizing the symposium, in particular Dr. Tengku Sifzizul Tengku Muhammad, Dr. Malik Mumtaz, and Ms. Diljit Kaur from Universiti Sains Malaysia. The assistance given by Maureen Twaig and Tom Merriweather at the Elsevier office is most gratefully acknowledged.

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SECTION I

Development and cell migration

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CHAPTER 1

Cell migration and evolutionary significance of GnRH subtypes

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Introduction

Gonadotropin-releasing hormone (GnRH, previously called luteinizing hormone-releasing hormone = LHRH) represents the first step in a cascade of events coordinating the complex physiology of reproduction and reproductive behavior in vertebrates. It was initially assumed that GnRH occurred as a single molecular form, in the septo-preoptic area of vertebrate brain, and that it served a single function. However, over the last decade, 13 structurally distinct forms of GnRHs have been isolated from different vertebrate species and two forms from invertebrates (Fig. 1). It has become increasingly clear that, all vertebrate species investigated to date possess two or three different GnRH forms, and that GnRH has multiple functions in addition to stimulating the release of gonadotropins (Parhar, 1997, 1999a; Sherwood et al., 1997; Weber et al., 1997). Recent research has also demonstrated the existence of GnRH receptor subtypes, which have evolved in conjunction with distinct GnRH ligands (Illing et al., 1999; Troskie et al., 2000; Wang et al., 2001; Parhar et al., 2002).

Studies using non-mammalian vertebrate species have played a central role in the discovery of molec-

ular diversity of GnRHs (Jimenez-Linan et al., 1997; King and Millar, 1997; Sherwood et al., 1997; Carolsfeld et al., 2000; Okubo et al., 2000a; Yoo et al., 2000) and the sites of embryonic origins of GnRH neurons (Muske, 1997; Parhar, 1997, 1999a). The multiplicity of GnRH and receptors has recently expanded with the discovery of chicken-II GnRH in the brain of humans and other mammals (Kasten et al., 1996; Lescheid et al., 1997; White et al., 1998; Gestrin et al., 1999; Urbanski et al., 1999). The present chapter summarizes neuroanatomical and biochemical studies from a comparative perspective with particular emphasis on the fish model to gain insights into the evolution and physiology of GnRH molecules and receptors.

Multiple GnRH genes

Some 30 years ago the structure of GnRH was first determined (Matsuo et al., 1971; Burgus et al., 1972). Since then, thirteen structurally distinct forms of GnRHs have been isolated from vertebrates and two from protochordates (Jimenez-Linan et al., 1997; Sherwood et al., 1997; Sower, 1997; Carolsfeld et al., 2000; Okubo et al., 2000a; Yoo et al., 2000). All known GnRH forms are ten amino acids in length (Fig. 1). The most common structural variation among the different forms of GnRHs occurs frequently in amino acid positions 5–8. However, the essential molecular sequences at the ends of the decapeptide, the 5'-pyroglutamyl-modified amino terminus and the 3'-amidated carboxy terminus have remained unchanged during

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	1	2	3	4	5	6	7	8	9	10	
TUNICATE I	pGLU	HIS	TRP	SER	ASP	TYR	PHE	LYS	PRO	GLY	NH2
TUNICATE II	pGLU	HIS	TRP	SER	LEU	CYS	HIS	ALA	PRO	GLY	NH2
LAMPREY I	pGLU	HIS	TYR	SER	LEU	GLU	TRP	LYS	PRO	GLY	NH2
LAMPREY III	pGLU	HIS	TRP	SER	HIS	ASP	TRP	LYS	PRO	GLY	NH2
DOGFISH	pGLU	HIS	TRP	SER	HIS	GLY	TRP	LEU	PRO	GLY	NH2
CHICKEN II	pGLU	HIS	TRP	SER	HIS	GLY	TRP	TYR	PRO	GLY	NH2
SALMON	pGLU	HIS	TRP	SER	TYR	GLY	TRP	LEU	PRO	GLY	NH2
CATFISH I	pGLU	HIS	TRP	SER	HIS	GLY	LEU	ASN	PRO	GLY	NH2
MEDAKA	pGLU	HIS	TRP	SER	PHE	GLY	LEU	SER	PRO	GLY	NH2
HERRING	pGLU	HIS	TRP	SER	HIS	GLY	LEU	SER	PRO	GLY	NH2
SEABREAM	pGLU	HIS	TRP	SER	TYR	GLY	LEU	SER	PRO	GLY	NH2
RANA	pGLU	HIS	TRP	SER	TYR	GLY	LEU	TRP	PRO	GLY	NH2
CHICKEN I	pGLU	HIS	TRP	SER	TYR	GLY	LEU	GLN	PRO	GLY	NH2
GUINEAPIG	pGLU	TYR	TRP	SER	TYR	GLY	VAL	ARG	PRO	GLY	NH2
MAMMAL	pGLU	HIS	TRP	SER	TYR	GLY	LEU	ARG	PRO	GLY	NH2

Fig. 1. Comparison of amino acid sequences of 15 GnRH forms. The amino acids printed in bold differ from the mammalian form (see Jimenez-Linan et al., 1997; King and Millar, 1997; Sherwood et al., 1997; Sower, 1997; Carolsfeld et al., 2000; Okubo et al., 2000a; Yoo et al., 2000).

evolution. Evidence suggests that in all the major vertebrate groups, chicken-II GnRH is the most highly conserved form, while the forebrain forms vary structurally in different species (King and Millar, 1997; Sherwood et al., 1997; Parhar, 1999a). The fact that more than one GnRH molecule also exists in protocordates (tunicates) and jawless fish (lampreys: Sherwood et al., 1997; Sower, 1997), strengthens the evidence that the presence of multiple forms of GnRH in a single species is an ancient pattern in evolution (Fig. 1).

The cDNAs encoding GnRH have been determined in mammals (Adelman et al., 1986), amphibians (Hayes et al., 1994), birds (Dunn et al., 1993) and bonyfish (Bond et al., 1991; Klungland et al., 1992; Suzuki et al., 1992; Bogerd et al., 1994; White et al., 1995; Gothilf et al., 1996). Since the original discovery in birds (Miyamoto et al., 1984), a cDNA encoding a second and a third GnRH was identified in teleosts (White et al., 1994, 1995; Gothilf et al., 1996). In recent years, more than one GnRH peptide and cDNA encoding GnRH have been sequenced from the brain of several fish species and placental mammals (tree shrew, humans: White et al., 1995, 1998; Gothilf et al., 1996; Kasten et al., 1996;

Gestrin et al., 1999). Genes encoding different GnRH forms have the same architecture of four exons separated by three introns and this has been highly conserved throughout evolution, despite changes in the size and sequences of exons and introns. In each gene, the first exon encodes the 5'-untranslated region; the second and third exons encode the signal peptide, GnRH decapeptide, a proteolytic cleavage site and the GnRH-associated peptide (GAP); and the fourth exon has the carboxy terminus of GAP and the 3'-untranslated region. Comparisons between the cDNA precursors encoding the distinct members of the GnRH family show complete conservation of the proteolytic cleavage site, high homology of the decapeptide, but high structural divergence in the GAP region (see King and Millar, 1997; Sherwood et al., 1997; Yu et al., 1997).

Phylogenetic distribution and ontogeny of GnRH subtypes

The brain of most vertebrate species has at least two but some advanced perciform fishes have three GnRH forms (White et al., 1995; Gothilf et al., 1996; Parhar, 1997). Localization studies have demon-

TELEOSTEAN RADIATION

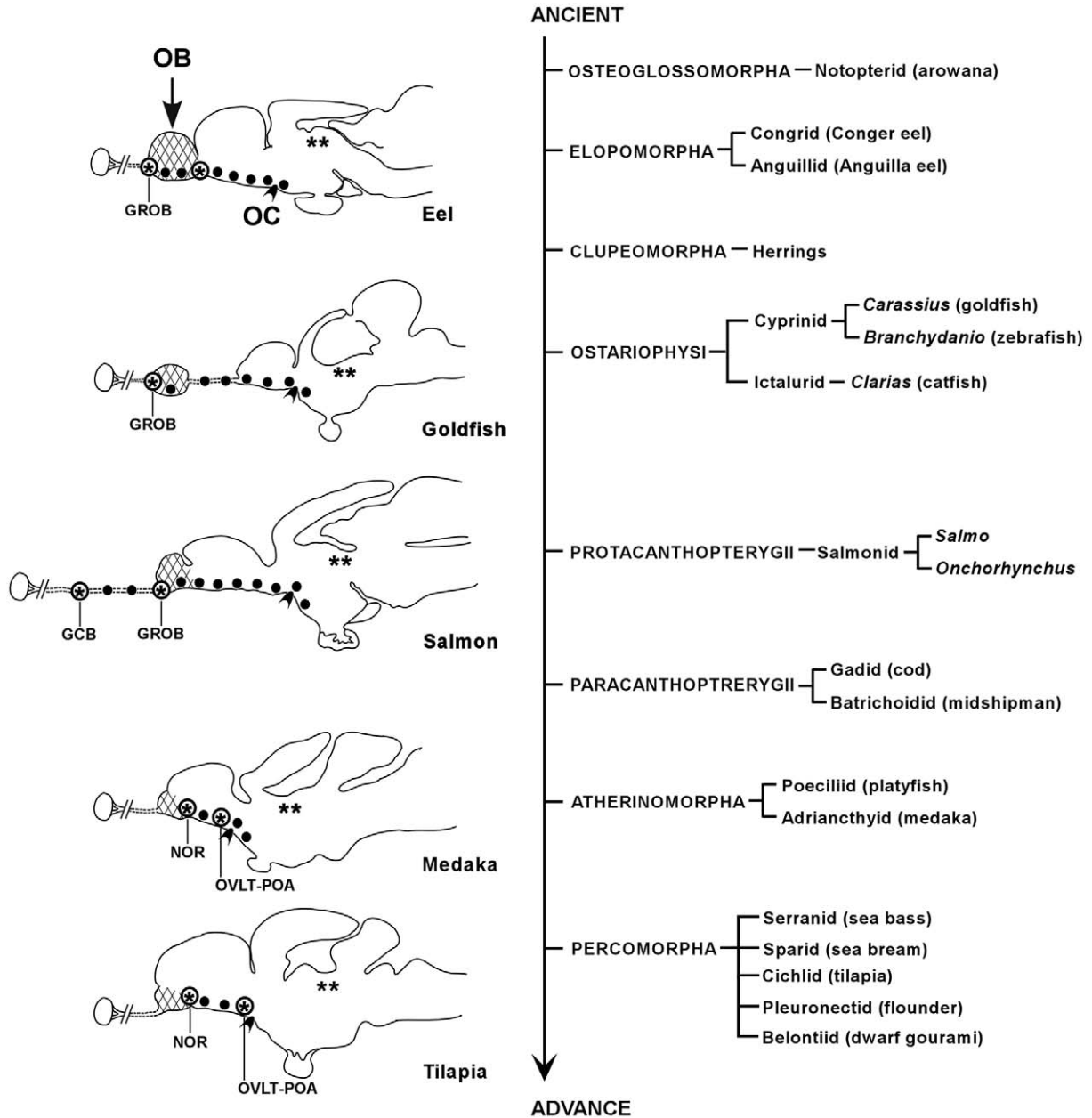


Fig. 2. Phylogenetic distribution of GnRHs in the teleostean fish radiation. Eel (Chiba et al., 1999), goldfish (Parhar et al., 2001), Salmon (Parhar and Iwata, 1993; Parhar et al., 1995, 1996), medaka (Parhar et al., 1998), Tilapia (Parhar, 1997). The evolutionary order of teleost (ancient to advanced) is modified from Meek and Nieuwenhuys (1998). OB, olfactory bulb; OC, optic chiasma; GROB, ganglia at the rostral olfactory bulbs; GCB, ganglia at the cribriform bone; NOR, nucleus olfactoretinalis; OVLT-POA cells synthesize medaka or seabream GnRH; dark dots represent scattered single GnRH cells; **, chicken-II GnRH cells in the midbrain. Note, with advancing phylogenetic order: GnRH ganglia shifts intracerebral to form the NOR; medaka and seabream GnRH seen in the OVLT-POA and a decrease in GnRH cells in the basal hypothalamus (nucleus tuberis lateralis).

strated differential distribution of GnRH molecular variants in distinct brain areas in vertebrates (Fig. 2). Comparative studies using different vertebrate species show remarkable similarity in the distribution pattern of GnRH cell bodies, fiber pathways and embryonic origins, suggesting that these systems are phylogenetically conserved (Muske, 1997; Sherwood et al., 1997; Parhar, 1999a). In the following paragraphs the patterns of distribution of GnRH subtypes in vertebrate species are described in some detail.

Cells of mesencephalic origin

The mesencephalic GnRH system is a highly conserved neuronal system present in teleosts, amphibians, reptiles, birds and mammals (see reviews by King and Millar, 1997; Muske, 1997; Sherwood et al., 1997; Parhar, 1999a). In the mesencephalon, GnRH cells are exclusively located at the caudal most part of the prosencephalon, rostral to the oculomotor nucleus. These cells lie along the midline, close to the subependyma of the third ventricle, and close to large blood vessels (Figs. 2 and 3). They are round to triangular in shape, about 30–40 μm

in diameter and synthesize chicken-II GnRH subtype (Parhar, 1997). The embryonic origin of mesencephalic GnRH neurons is different from the forebrain GnRH neurons. In the urodele amphibians, ablations of the olfactory placodes results in bilateral loss of GnRH neurons in the hypothalamus but not chicken-II GnRH neurons in the midbrain; demonstrating that mesencephalic GnRH neurons do not originate from the olfactory placodes but from the ventricular ependyma of the third ventricle (Northcutt and Muske, 1994). Similarly, developmental studies in fishes have shown that chicken-II GnRH neurons differentiate from precursor cells in the ventricular ependyma (Parhar and Iwata, 1993; Parhar, 1997; Parhar et al., 1998) (Fig. 3), which might also be true for other vertebrate species.

Cells of olfactory origin

Ancient migratory route (olfactory to diencephalon)

Land vertebrates

The embryonic origin of GnRH neurons from the olfactory placode and their subsequent migration into

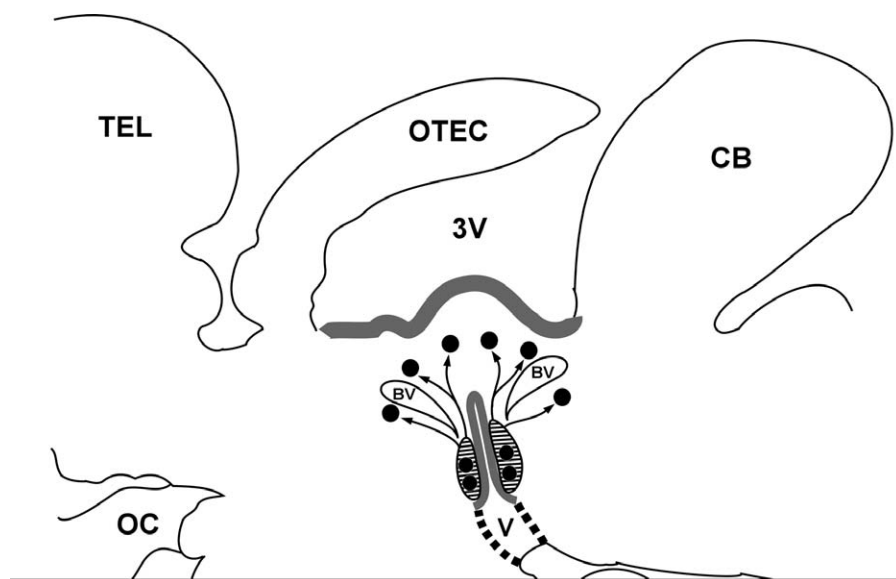


Fig. 3. Diagrammatic representation of sagittal brain section illustrating the location of midbrain chicken-II GnRH neurons in tilapia *Oreochromis niloticus*. Chicken II GnRH synthesizing neurons originate from the ventricular ependyma (shaded area). These cells migrate a short distance and are seen located around large blood vessels (Parhar and Iwata, 1993; Parhar, 1997). Dark dots represent chicken-II GnRH neurons. Tel, telencephalon; OTEC, optic tectum; CB, cerebellum; OC, optic chiasma. BV, blood vessel; V, ventricle.

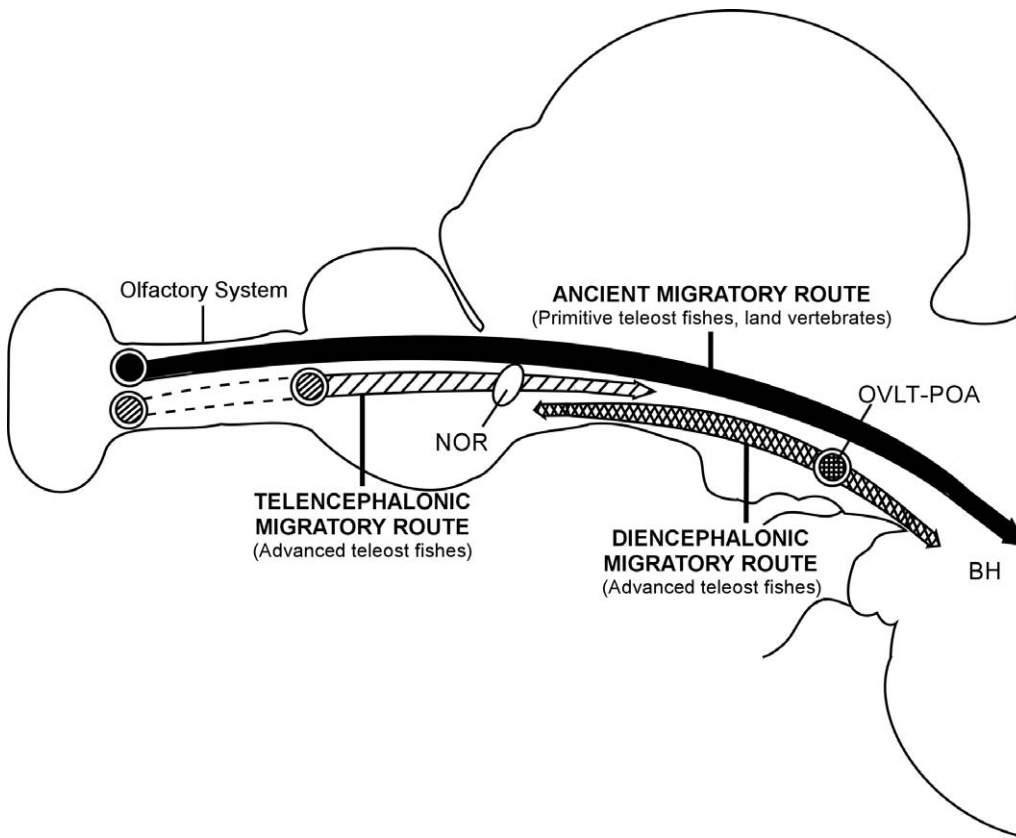


Fig. 4. Diagrammatic representation of a sagittal brain section illustrating the migratory paths taken by GnRH neurons. In primitive teleosts (salmon, catfish, mammalian GnRH) and in land vertebrates (chicken-I, mammalian GnRH) GnRH neurons migrate along the ‘*ancient migratory route*’, which originates from the olfactory placodes and extends to the diencephalon–basal hypothalamus (BH). Cells are seen scattered but as a continuum along this path. In advanced teleosts, salmon GnRH synthesizing neurons migrate along the ‘*telencephalonic migratory route*’, which originates from the olfactory placodes (or olfactory system) and extends to the nucleus olfactorectinalis (NOR), with scattered cells along the basal telencephalon. In advanced teleosts (medaka, tilapia) GnRH cells synthesizing medaka GnRH and seabream GnRH originate from the diencephalonic regions (OVLT–POA, organum vasculosum lamina terminalis–preoptic area) and probably migrate along the ‘*diencephalonic migratory route*’.

the forebrain–preoptic area is unique among brain cells (Fig. 4). This migratory route, which I describe here as the ‘*ancient migratory route*’ has been recorded in mammals including humans (Schwanzel-Fukuda et al., 1989; Schwanzel-Fukuda, 1997) and in non-mammalian vertebrate species (birds: Murakami et al., 2000; amphibians: D’Aniello et al., 1994; Muske, 1997; fishes: Parhar and Iwata, 1993; Parhar et al., 1995; Parhar, 1997). In virtually all land vertebrates, GnRH neurons in the forebrain of adults appear diffusely distributed within the diencephalic region. Majority of the GnRH cells are present in the septo-preoptic area as a loose cluster of disperse cells

with few scattered cells in the basal hypothalamus (Silverman et al., 1994). In rodents and birds, GnRH neurons migrating from the olfactory placodes are strongly associated with fibers immunoreactive for cell adhesion molecules (polysialylated form of neural cell adhesion molecule, PSA-NCAM: Murakami et al., 2000; Wray, 2001). Microinjections of antibodies against NCAM into the olfactory placodes in mice (Schwanzel-Fukuda, 1997) or ablations of the olfactory placodes in amphibians and birds (Murakami et al., 1992; Northcutt and Muske, 1994) result in the loss of preoptic GnRH neurons. Therefore, these studies provide strong evidence that the

embryonic origin of the septo-preoptic GnRH neurons is the olfactory placodes.

Primitive teleost fishes

In teleost fishes, which belong to the evolutionary primitive order (e.g. salmonids), GnRH neurons follow the ‘*ancient migratory route*’ similar to that in land vertebrates (Fig. 4). During embryonic development, neurons that synthesize salmon GnRH are born in the basal regions of the olfactory placodes (Parhar and Iwata, 1993; Parhar et al., 1995). Small groups of cells are seen outside the central nervous system as cell clusters in the olfactory nerve close to the olfactory epithelium and at the interface of the olfactory nerve and the olfactory bulbs. These clusters of GnRH cells were described as ganglia at the cribriform bone (GCB) and ganglia at the rostral olfactory bulbs (GROB) in the salmonids for the first time (Parhar and Iwata, 1993; Parhar et al., 1994, 1995) (Fig. 2). The arrival of GnRH neurons in the basal telencephalon coincides with the completion of smoltification in salmonids (Parhar et al., 1994, 1995; Parhar and Iwata, 1996) and metamorphosis in amphibians (Hayes et al., 1994). In adults, scattered GnRH cells are seen along the basal regions of the olfactory forebrain; forming a continuum from the medial parts of the olfactory nerve (terminal nerve), basal olfactory bulbs, basal telencephalon, preoptic area and the basal hypothalamus (nucleus lateralis tuberis). No GnRH cell clusters are seen within the olfactory bulb-forebrain at any developmental stage. This pattern of GnRH cellular distribution is most prevalent in teleost fish of the order elopomorpha, clupeomorpha, ostariophysi and protacanthopterygii, which include species such as eels (Montero et al., 1994; Chiba et al., 1999), goldfish (Kim et al., 1995; Parhar et al., 2001), catfish (Zandbergen et al., 1995) and salmonids (Parhar and Iwata, 1993, 1996; Parhar et al., 1994, 1995) (Fig. 2). Interestingly, these species have long olfactory nerves (salmonids) and/or displaced olfactory bulbs separated from the telencephalon by long olfactory tracts (goldfish, catfish). These primitive teleosts have been reported to have only two GnRH forms, i.e. chicken-II GnRH along with salmon GnRH (salmonids, goldfish) or catfish GnRH (catfish) or mammalian GnRH (eel) (King and Millar, 1997; Sherwood et al., 1997).

Thus, in primitive teleost fishes the distribution pattern, migratory route and the placodal origins of forebrain GnRH (salmon, catfish GnRH) is similar to mammalian and chicken-I GnRH in land vertebrates.

Telencephalonic migratory route (olfactory to telencephalon)

Advance teleost fishes

The evolutionarily advanced teleost fishes (atherinomorpha: medaka; percomorpha: tilapia) lack the ‘*ancient migratory route*’, instead they have devised an alternate GnRH migratory route which consists of a telencephalonic and a diencephalonic component (Fig. 4). The diencephalonic component gives birth to GnRH neurons seen in the organum vasculosum of the lamina terminalis–preoptic area (OVLT–POA). The ‘*telencephalonic migratory route*’, comprises GnRH neurons born in the olfactory placodes, and migrate a short distance along the olfactory nerves–basal olfactory bulbs to form the nucleus olfactoretinalis (NOR) at the caudalmost part of basal olfactory bulbs, with scattered neurons in the rostro-basal telencephalon (Fig. 4). In fact, like the ‘*ancient migratory route*’, the ‘*telencephalonic migratory route*’ has its embryonic origins in the olfactory placodes. However, in highly evolved teleosts (e.g. perciform tilapia), the olfactory nerve is short and therefore during development the presumptive olfactory bulbs and the olfactory placodes lie against each other, which gives the impression that GnRH neurons originate from or begin GnRH synthesis when they reach the rostral olfactory bulbs (Parhar, 1997). In advanced teleosts, the most distinctive feature of forebrain GnRH neuronal distribution is the clustering of GnRH neurons at the caudalmost part of the olfactory bulb–telencephalon interface to form the nucleus olfactoretinalis (NOR: Munz and Claas, 1987) (Fig. 2). Furthermore, compared to primitive teleost fishes, advanced teleosts have fewer scattered neurons along the basal olfactory bulbs, basal telencephalon and the basal hypothalamus (nucleus lateralis tuberis). In fact, cells in the basal hypothalamus appear to decrease in number with advancing evolutionary order. This pattern of GnRH cellular distribution is most prevalent in the teleostean order paracanthopterygii (midshipman: Grober et al.,

1995), atherinomorpha (platyfish: Schreibman and Margolis-Nunno, 1987; medaka: Parhar et al., 1998) and the more advanced percomorpha (tilapia: Parhar, 1997) (Fig. 2). Interestingly, these species have short olfactory nerves and their olfactory bulbs are located against the telencephalic hemispheres. These fishes have been reported to have three GnRH forms, i.e. preoptic GnRH (seabream GnRH: tilapia niloticus, haplochromis burtoni; medaka GnRH: medakafish); mesencephalic GnRH (chicken-II GnRH) and telencephalic GnRH (salmon GnRH: tilapia, *H. burtoni*, medaka) (White et al., 1995; Gothilf et al., 1996; Parhar, 1997; Okubo et al., 2000a).

When compared along the teleostean evolutionary radiation, salmon GnRH has two distinct distribution patterns: (1) olfactory system to diencephalon, an ancient distribution pattern seen in primitive fishes; (2) olfactory system to telencephalon, an advanced distribution pattern seen in more recently evolved teleost fishes (Fig. 4).

Cells of diencephalic origin

Unlike primitive teleosts, advanced teleosts have a distinct loose cluster of GnRH cells in the antero-basal OVLT–POA area, with few scattered cells in the basal telencephalon and the basal hypothalamus (nucleus lateralis tuberis). These cells synthesize seabream GnRH (cichlid: White et al., 1995; tilapia: Parhar, 1997; Red seabream: Okuzawa et al., 1997; Gilthead seabream: Gothilf et al., 1996) or medaka GnRH (medakafish: Okubo et al., 2000a). They are about 20–25 μm in diameter, with unipolar or bipolar dendrites and number about 100–200 cells in cichlid fish. This OVLT–POA cell population, unlike the nucleus olfactoryretinalis (NOR) of the ‘telencephalic migratory route’, does not originate from the olfactory placode; instead this cell population originates from the basal diencephalons (Parhar, 1997; Parhar et al., 1998). The OVLT–POA GnRH cells appear to migrate dorsolaterally from the basal diencephalic regions, along what might be considered the ‘diencephalic migratory route’ (Fig. 4). As in the advanced teleost fishes, the origin of OVLT–POA GnRH neurons from the basal diencephalon has been observed in some species of birds and lampreys (Norgren and Chen, 1994; Tobet et al., 1996). However, a diencephalic origin

of forebrain GnRH neurons remains to be seen in mammals.

Two GnRH forms in the forebrain of advanced teleosts

Telencephalic versus diencephalic GnRH neurons

Morphological and biochemical evidence

A large body of published evidence shows that in advanced teleost fishes (paracanthopterygii, atherinomorpha, percomorpha), GnRH cells of the terminal nerve ganglia (nucleus olfactoryretinalis, NOR) are distinct from cells of the OVLT–POA.

(1) At all developmental stages, morphologically cells of the NOR are large, round in shape ($\sim 30\text{--}40\ \mu\text{m}$) and present as clusters (ganglia) whereas the POA neurons are small and usually fusiform in shape ($\sim 20\text{--}30\ \mu\text{m}$) and scattered (Parhar, 1997; Parhar et al., 1998).

(2) The cDNA sequence of NOR specific salmon GnRH is different from the OVLT–POA specific seabream or medaka GnRH (seabream GnRH: White et al., 1995; Gothilf et al., 1996; medaka GnRH: Okubo et al., 2000a).

(3) In many species of teleost fish, the NOR but not the OVLT–POA GnRH neurons co-express molluscan cardioexcitatory tetrapeptide (FMRFamide: Stell et al., 1984). Furthermore, NOR begins GnRH expression during early embryonic life, long before gonadal development, and these cells are regulated by thyroid hormones and testosterone (Soga et al., 1998; Parhar et al., 2000). On the contrary, the expression of OVLT–POA GnRH coincides with gonadal sex differentiation (Parhar, 1997; Chiba et al., 1999), and these cells are regulated by estrogen and ketotestosterone (Soma et al., 1996; Parhar et al., 2000).

Developmental evidence

There is compelling evidence that the NOR and the OVLT–POA GnRH neurons have embryonic origins from progenitor cells situated at different locations. Developmental studies have shown that the salmon GnRH synthesizing NOR neurons originate

from the olfactory placodes whereas the seabream GnRH synthesizing OVLT–POA neurons originate from the anteriopasal OVLT–POA region in the perciform tilapia and possibly medaka GnRH in the medakafish (Parhar, 1997; Parhar et al., 1998). Supportive evidence from studies in the lampreys and birds suggests that the POA GnRH neurons originate from the basal diencephalon (Norgren and Chen, 1994; Tobet et al., 1996). Furthermore, some species of lampreys lack a terminal nerve and terminal nerve GnRH neurons but not the POA GnRH neurons (Eisthen and Northcutt, 1996).

Hence, in advanced teleost fishes, morphological and biochemical studies demonstrate that the NOR and the OVLT–POA are two different GnRH cell populations in the forebrain, which synthesize distinct GnRH molecular forms and have different embryonic origins; from the olfactory placodes and within the diencephalons respectively. Likewise, is there evidence of two GnRH forms in the forebrain of other vertebrates as in advanced teleost fishes?

Evidence of a third GnRH form in land vertebrates?

Like in advanced teleost fishes there is evidence of two GnRH forms in the forebrain of primitive teleost fishes and land vertebrates. Two GnRH forms have been cloned from the forebrain of primitive fishes, such as the goldfish, catfish and salmonids. However, tetraploidy has been cited as a cause of this gene duplication (Ashihara et al., 1995; Yu et al., 1997). In mammals, there is physiological evidence of more than one population of forebrain GnRH neurons. For example, under certain physiological states only a subpopulation of POA GnRH neurons express galanin (Merchenthaler et al., 1991) or c-Fos (Lee et al., 1990), and not all GnRH neurons of the septo-preoptic area are under gonadal steroid influence (Herbison, 1998). The existence of two distinct cell populations in the forebrain, responsible for LH surge and pulse generation, has been long debated (Kimura and Funabashi, 1998). Equally debated is the issue of LH and FSH control by one or two different GnRH molecules. Recently, however, McCann and coworkers have shown that Lamprey III GnRH can function, as a FSH releasing hormone (Yu et al., 1997) and LH release is

the classical role of mammalian GnRH (= LHRH). Lamprey III GnRH has been localized in the forebrain of rats and mice (Hiney et al., 2002; Ogawa et al., 2002a), which further emphasizes the existence of more than one GnRH form in the forebrain. However, the site(s) of embryonic origin of Lamprey III GnRH is unknown, which could be the diencephalon. Furthermore, new populations of GnRH neurons have been reported in the forebrain whose origin does not appear to be placodal (Skynner et al., 1999). Thus, unlike advanced teleost fishes with two distinct GnRH molecular forms in the forebrain, land vertebrates, on the other hand, lack specific markers to allow distinction between forebrain GnRH neurons of placodal origin and subpopulations of POA GnRH neurons that might have origins in the basal diencephalon.

GnRH receptor subtypes

GnRH exerts diverse intracellular actions through binding to specific G-protein coupled receptors (Sealfon et al., 1997). The existence of several molecular forms of GnRH ligand within a single species of bonyfish and their ability to effectively stimulate gonadotropins, growth hormone, prolactin and somatotactin cells (Parhar and Iwata, 1994; Melamed et al., 1996; Weber et al., 1997) suggests that there may also be more than one form of GnRH receptor (Troskie et al., 1998). A single class of GnRH receptor has been described in some teleost species (African catfish: Blomenrohr et al., 1997; Tensen et al., 1997; striped bass: Alok et al., 2000; rainbow trout: Madigou et al., 2000) but two different class of GnRH receptors have been demonstrated in the goldfish (Illing et al., 1999), medaka (Okubo et al., 2000b), primates (Millar et al., 2001; Neill et al., 2001), which express two different mRNA. More recently, a third GnRH receptor subtype has been reported in the bullfrog (Wang et al., 2001).

In the pituitary of perciform tilapia, GnRH-Rs type IA and IB have been shown in LH cells, GnRH-R type IB in prolactin cells and GnRH-R type III in growth hormone cells. The presence of type IA, IB and type III GnRH receptors in different cells in the perciform tilapia, demonstrates that three types of GnRH-Rs exist in a single diploid species (Parhar et al., 2002). This supports the notion that the three

native GnRH variants in tilapia might have their respective cognate receptors. The presence of type IA and IB GnRH-Rs in FSH and LH cells suggests that two different GnRH ligands can independently control the synthesis/secretion of FSH and LH. Alternatively, dimerization of type IA and IB GnRH-Rs might be important for their functional activation, which implies a complex interplay of GnRH ligands and receptors.

Nomenclature of GnRH subtypes

Based on DNA sequences Fernald and White (1999) classified GnRH subtypes into: hypothalamic form (Type I); mesencephalonic form (Type II) and telencephalonic form (Type III). The classification of salmon GnRH as telencephalonic form (GnRH Type III) by Fernald and White (1999) does not take into account the distribution of salmon and catfish GnRH in primitive fishes, which is not restricted to the telencephalon but is also present in the hypothalamus. On the contrary, 'salmon GnRH' in advanced teleost fishes has a different molecular structure and distribution pattern from salmon GnRH in primitive fishes (Parhar et al., 1996) (Figs. 2 and 4). Therefore, based on the distribution pattern and ontogenic origins from the olfactory system it would be better to consider: (1) salmon and catfish GnRH of primitive fishes along with mammalian GnRH as GnRH-I; (2) the highly conserved mesencephalonic GnRH as GnRH-II; (3) 'salmon GnRH' of advanced teleost as GnRH-III; (4) the newly cloned medaka and seabream GnRH, present only in recently evolved teleost fishes, as GnRH-IV (Fig. 5).

Functional significance of GnRH subtypes

Cells of olfactory origin (olfactory to diencephalon): hypophysiotropic

Primitive teleost fishes and land vertebrates (GnRH-I)

In the following discussion, based on the distribution pattern and their olfactory-related origins, salmon GnRH of primitive fishes, catfish, chicken-I and mammalian GnRH are grouped as GnRH-I. GnRH-I is the only GnRH form present in the forebrain of

land vertebrates and primitive fishes. The regulation of gonadotrophs (LH/FSH) by GnRH-I is well documented in all vertebrate species (Sherwood et al., 1997; Yu et al., 1997). In primitive teleost fishes, the synthesis of GnRH-I (salmon GnRH: salmonids, Parhar and Iwata, 1994; Parhar et al., 1995; catfish GnRH: catfish, Dubois et al., 2000; mammalian GnRH: eel, Chiba et al., 1999) prior to the development of gonadotrophs, the presence of GnRH-I immunoreactive fibers in the pituitary and GnRH Type IA receptors specifically in the gonadotrophs supports the role of GnRH-I in the differentiation and regulation of the pituitary gonadotrophs and gonads (Parhar et al., 2002). Similarly, in land vertebrates, GnRH-I (mammalian GnRH, chicken-I GnRH) plays a vital role in the regulation of LH synthesis-secretion (Silverman et al., 1994; King and Millar, 1997). In fishes, as in land vertebrates, gonadal status and gonadal hormones are known to regulate GnRH-I activity (Gore and Roberts, 1997; Dubois et al., 1998; Herbison, 1998). In addition, thyroid hormones have been implicated in the regulation of GnRH-I during metamorphosis in salmonids (Parhar et al., 1994, 1996; Parhar and Iwata, 1996). In humans, the failure of neurons synthesizing GnRH-I to migrate from the olfactory placodes to the basal forebrain results in Kallman's Syndrome, which is hypogonadotropic hypogonadism coupled with anosmia (Schwanzel-Fukuda, 1997). That GnRH-I is involved in reproduction as well as in imprinting of odors and olfactory memory has been speculated in downstream migrating salmonids (Parhar et al., 1994, 1995; Parhar and Iwata, 1996).

Cells of mesencephalonic origin: neuromodulator

All vertebrate species (GnRH-II)

GnRH-II has remained unchanged during evolution and therefore is identical in all vertebrate species studied to date (King and Millar, 1997). Nevertheless, the function of GnRH-II is still speculative. GnRH-II may have originated as a regulator of reproduction; however, during evolution it has also acquired non-reproductive and extra-pituitary functions. For example, within the central nervous system the widespread distribution of GnRH-II fibers suggests it might act as a neuromodulator and perhaps

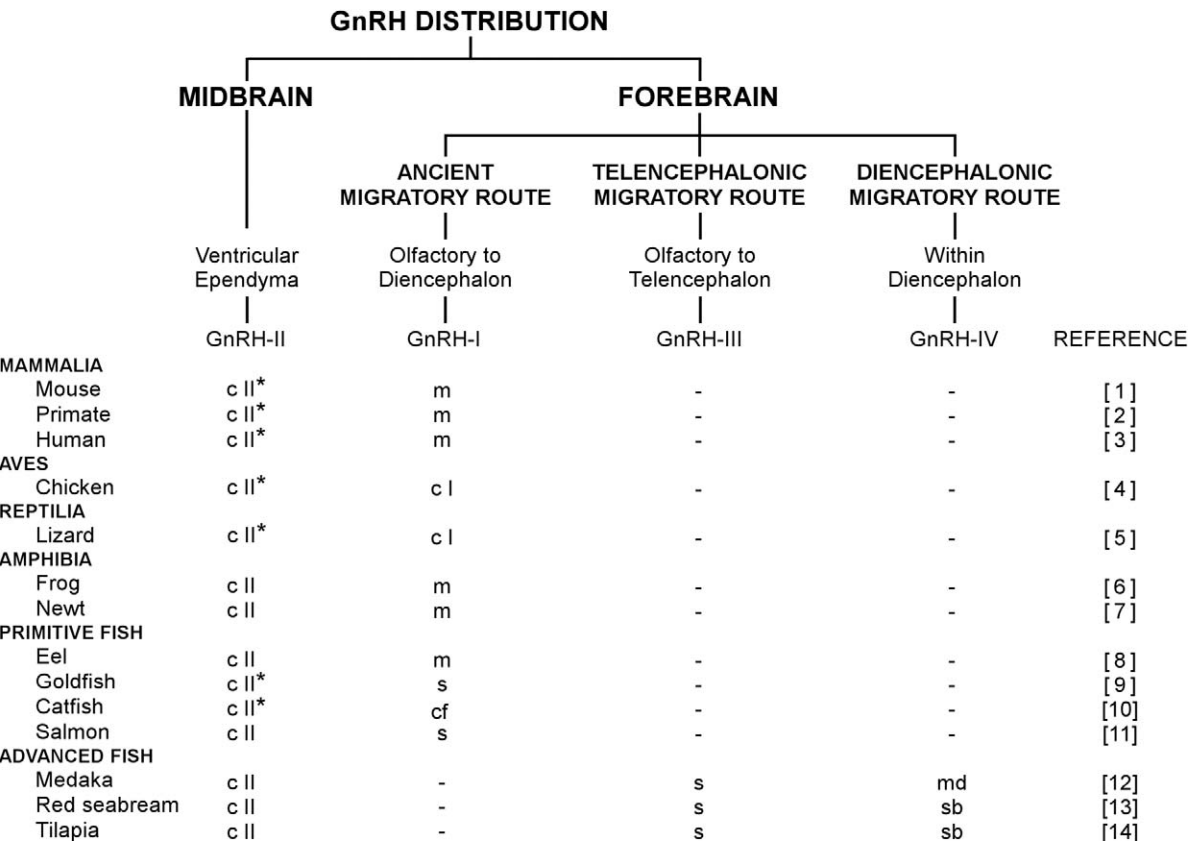


Fig. 5. The proposed nomenclature of GnRH (GnRH I–IV) is based on the sites of origin, migration and final location of GnRH cells in the midbrain and forebrain. Chicken II GnRH (cII) is the most conserved form present in the midbrain of all vertebrates. The seabream (sb) and medaka (md) GnRH forms are present in the diencephalon–preoptic area of only advanced teleost. Asterisks indicates not studied but most likely of ventricular origin. Dashed lines indicate unknown/uncertain. S, salmon GnRH; cI, chicken-I GnRH; cf, catfish GnRH; m, mammalian GnRH. References: [1] Schwanzel-Fukuda, 1997; Wray, 2001; [2] Terasawa and Quanbeck, 1997; [3] Schwanzel-Fukuda et al., 1989; [4] Murakami et al., 2000; [5] D’Aniello et al., 1994; [6] Hayes et al. (1994); [7] Murakami et al., 1992; [8] Chiba et al., 1999; [9] Parhar et al., 2001; [10] Dubois et al., 1998; [11] Parhar and Iwata, 1993; Parhar et al., 1995; [12] Parhar et al., 1998; [13] Okuzawa et al., 1997; [14] Parhar, 1997.

fine-tune reproductive behaviors. In the musk shrew, a placental mammal, and the ringdove GnRH-II has been implicated in mating and courtship behaviors (Rissman, 1996; Silver et al., 1996). On the contrary, gonadal hormones have no effect on GnRH-II mRNA and peptide levels, which suggests that GnRH-II might be under different regulatory mechanism (Soga et al., 1998; Parhar et al., 2000) and might have functions other than reproduction. In fishes, GnRH-II regulates prolactin release (Weber et al., 1997) probably via Type IB GnRH receptors (Parhar et al., 2002), which suggests a possible role of GnRH-II in osmoregulation/acid-base balance.

Cells of olfactory origin (olfactory to telencephalon): hypophysiotropic, neuromodulation and reproductive behavior

Advance teleost fishes (GnRH-III)

Compared to primitive teleost fishes, ‘salmon GnRH’ in advanced teleost fishes has a different molecular structure and is synthesized specifically by cells of the nucleus olfactoretinalis (NOR, Parhar et al., 1996). The presence of GnRH-III immunoreactive fibers in the pituitary suggests its role as a hypophysiotropic hormone (Parhar et al., 1996; Parhar, 1997),

and the widespread fibers in the brain suggests its role as a neuromodulator (Oka, 1997). Sex steroids and thyroid hormones have been implicated in the regulation of GnRH-III (Soga et al., 1998; Parhar, 1999b; Parhar et al., 2000). More recently, the role of GnRH-III, has been extended to include aggression and nest building behavior (Yamamoto et al., 1997; Soga et al., 2001; Akiyama et al., 2002; Ogawa et al., 2002b).

Cells of diencephalonic origin: hypophysiotropic hormone

Advance teleost fishes (GnRH-IV)

GnRH-IV (seabream, medaka GnRH) is present in the OVLT–POA area, only in advanced teleost fishes (cichlids: White et al., 1995; tilapia: Parhar, 1997; seabream: Gothilf et al., 1996; Okuzawa et al., 1997; medakafish: Okubo et al., 2000a). The concurrent development of GnRH-IV synthesizing neurons, GnRH fibers in the pituitary, gonadal sex differentiation and the appearance of steroid producing cells in the gonads, suggest GnRH-IV may have a role in sex differentiation (Parhar, 1997). GnRH-IV is known to regulate LH/FSH in advanced teleost fishes (Holland et al., 1998). In addition, an increase in GnRH-IV cell size and cell numbers is associated with gonadal maturation and changes in sex steroid levels (estrogen: Parhar et al., 2000; testosterone: Soga et al., 1998; Ketotestosterone: Soma et al., 1996), which further emphasizes the role of GnRH-IV in reproduction. Furthermore, reproduction-related territorial behavior has been associated with change in GnRH-IV neuronal size (Francis et al., 1993). Since advanced teleost fishes are the only vertebrate species to possess GnRH-IV, therefore, it is likely that this GnRH molecular form might have been acquired for functions specific to advanced teleost.

Summary

Hypothetically it can be assumed that in advanced teleost fishes, GnRH-III and GnRH-IV neurons migrate along the ‘telencephalonic’ (anterior) and ‘diencephalonic’ (posterior) migratory route, which perhaps fuses in primitive teleost fishes and land vertebrates to form the ‘ancient migratory route’ (in

all probability = *nervus terminalis*; see Von Bartheld et al., 1988) of GnRH-I neurons.

The difference in distribution pattern of GnRH forms in the vertebrate brain is due to distinct embryonic origins:

(1) *Cells of olfactory origin*, which give rise to GnRH-I (salmon, catfish, chicken I, mammalian GnRH) are distributed along the olfactory system and the basal forebrain in primitive fishes and in land vertebrates; GnRH-I might be pivotal for LH/FSH synthesis–release, olfaction and metamorphosis in lower vertebrates. In advanced teleost fishes, neurons synthesizing GnRH-III (‘salmon’ GnRH) originate from the olfactory system; they are distributed along the basal olfactory bulbs, with distinct ganglia (NOR) at the caudalmost part of the olfactory bulbs and few scattered cells in the basal telencephalon. The NOR might function as a neuromodulator, hypophysiotropic hormone and regulate visual associated reproductive behaviors.

(2) *Cells of mesencephalonic origin*, which give rise to GnRH-II (chicken-II GnRH) are evolutionarily conserved; might function as a neuromodulator involved in motor-associated reproductive behaviors and acid–base balance.

(3) *Cells of diencephalonic origin*, which give rise to GnRH-IV (seabream, medaka GnRH); they are localized in the anterior-basal OVLT–POA area and present only in advanced teleost fishes. GnRH-IV has been implicated in gonadal sex differentiation, gonadal maturation, LH/FSH secretion and territorial behavior. Advance teleost fishes for yet unknown functions might have acquired GnRH-IV.

Although all GnRH subtypes participate in some aspect of reproduction; the precise function of each GnRH form still remains unclear.

Acknowledgements

I would like to thank my students, T. Soga, S. Yamada, H. Tosaki and S. Ogawa for their invaluable help.

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CHAPTER 2

GnRH in the invertebrates: an overview

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Introduction and comparative historical perspective

The gonadotropin-releasing hormone [GnRH = luteinizing hormone-releasing hormone (LHRH)] represents a pivotal peptide in animal reproduction. GnRH is a decapeptide originally isolated from the porcine and ovine hypothalamus and characterized for its ability to enhance the release of pituitary gonadotropins, FSH and LH (Amoss et al., 1971; Matsuo et al., 1971). This pioneering research has led to the identification of at least 11 different forms of GnRH in vertebrates, 2 new forms in tunicates, and 1 in a mollusk (see Powell et al., 1996; Jimenez-Linan et al., 1997; Carolsfeld et al., 2000; Iwakoshi et al., 2002). These GnRH forms can be identified as follows: mammalian (mGnRH), guinea pig GnRH, chicken-I (cGnRH-I), chicken-II (cGnRH-II), salmon (sGnRH), seabream (sbGnRH), catfish (cfGnRH), herring (hrGnRH), dogfish (dfGnRH), lamprey-I (lGnRH-I), lamprey-III (lGnRH-III), tunicate-I (tGnRH-I), tunicate-II (tGnRH-II, a disulfide-linked dimer), and octopus (octoGnRH, a dodecapeptide). Based on amino acid homology, i.e., sharing part of their sequence, GnRHs in the animal kingdom are now represented as a family of structurally related peptides. All known

forms of GnRH peptides have in common a pyroglutamyl residue at the amino terminus and an amidated glycine at the carboxy terminus. Except for the octoGnRH, all other known forms are decapeptides and differ by one or more amino acids; amino acids 4 and 9 are conserved in all forms known to date.

Since it triggers the release of pituitary gonadotropins in all vertebrates, its current denomination as 'GnRH' is fully justified. During the late 1960s and early 1970s, the isolation of a hypothalamic hypophysiotropic peptide, its characterization as GnRH, and the determination of its amino acid sequence, marked the beginning of an era in GnRH research. Since that time many investigators have focused their attention to what seems to be an ever increasing number of its molecular variants, their distribution in the neural and nonneural tissues, and their physiological roles as neurotransmitter, neuromodulator, neurohormone, local hormone, and so forth, placing the GnRH at an important junction between endocrine and nervous systems.

Since the late 1970s to date, a vast assortment of review articles and brief updates has been published: Silverman and Zimmerman (1978), Nozaki and Kobayashi (1979), King and Millar (1980, 1987, 1991, 1992, 1995, 1997), Peter (1983), Nozaki et al. (1984), Demski (1984, 1987), Barry et al. (1985), Sherwood (1987), Silverman (1988), Clayton (1989), Chieffi et al. (1991), Andersen et al. (1992), Muske (1993, 1997), Sherwood et al. (1993, 1997), Silverman et al. (1994), Rastogi and Iela (1994), Parhar et al. (1995), Rissman (1997), Demski et

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al. (1997), Kim et al. (1997), Rastogi et al. (1998). These have dealt variously with the distribution of GnRH-like immunoreactivity in the central nervous system (CNS) and nonneural tissues, primary structure of GnRH-like molecule, phylogenetic diversity, endocrine/nonendocrine and behavioral regulation of GnRH synthesis and secretion (also see Rissman, 1996; D'Aniello et al., 2001), genes encoding GnRHs, GnRH neuronal migration from olfactory placode into the developing basal forebrain in jawed vertebrates, bioactivity, mechanisms of action of GnRH and its receptors, regulation of GnRH gene expression, and its evolution either within a specific group of animals or across the entire vertebrate and/or invertebrate lineage. In the early 1970s attention was projected to higher vertebrates (particularly mammals, and less frequently birds). Research on GnRH-like peptides in nonmammalian vertebrates, including reptiles, amphibians and fishes, gained momentum during the late 1970s, and it was in the 1980s that the term GnRH was largely accepted to replace LHRH, at least in nonmammalian vertebrates. During a time span of little more than 30 years, little over 100 species of vertebrates (17 species of mammals, 8 birds, 18 reptiles, 22 amphibians, little over 45 bony, cartilaginous and agnathan fishes) and only 17 species of invertebrates have been examined (see Demski et al., 1997; King and Millar, 1997; Parhar, 1997; Sower, 1997; Carolsfeld et al., 2000). The invertebrates were totally ignored until 1980 when Georges and Dubois, for the first time, ventured into the realm of protochordates and observed GnRH-like immunoreactivity in the nervous system of an ascidian tunicate, *Ciona intestinalis* (Georges and Dubois, 1980). This finding, however, did not provide an impetus strong enough to proceed with active research on the presence of GnRH-like peptides in the invertebrates. However, with the availability in successive years of more sophisticated instrumentation and biochemical methodology, and the understanding that GnRH is a molecule not restricted to the nervous system, a greater attention was focused on invertebrates during the last 6–7 years. These studies have provided surprising results (see Powell et al., 1996; Di Fiore et al., 2000; Zhang et al., 2000; Iwakoshi et al., 2002), in as much as in some invertebrates vertebrate-type GnRH forms have been isolated and characterized.

Immunocytochemical localization, isolation and characterization of GnRH-like peptides

Cnidarian nervous system

According to a recent study, using anti-cGnRH-II and anti-mGnRH, on the sea pansy (*Renilla koellikeri*) and starlet sea anemone (*Nematostella vectensis*) by Anctil (2000), GnRH-immunoreactive (GnRHir) neurons are distributed individually in the endodermal and myoepithelial layers throughout, as well as in mesenteric filaments bearing gametes. In the mesenteric filaments such neurons usually occur in clusters. In the sea pansy, GnRHir neurons are relatively more abundant in the tentacles, and those located in the wall of the gastrovascular cavity often possess a cilium. In the starlet anemone, on the other hand, GnRHir fibers innervate the follicular layer of the ovocytes. All GnRHir neurons in the two species represent a subset of neurons sharing similarities of morphology, distribution and innervation targets. High-performance liquid chromatography (HPLC) and radioimmunoassay (RIA) of whole sea pansy extracts yielded two elution peaks which were immunoreactive with antisera against dfGnRH or mGnRH (Anctil, 2000).

Flatworms

No reports are available.

Nematodes

Brownlee et al. (1993) made a brief mention of the presence of GnRH-like immunoreactivity in some parts of the central nervous system of the roundworm, *Ascaris suum*.

Annelids

Dhainaut-Courtois et al. (1985) failed to observe GnRH-like immunoreactivity in the polychaet worm, *Nereis diversicolor*. However, as cited in Al-Yousuf (1990), GnRHir material is present in the neural tissue of the leech, and in some nonneural tissues of earthworms.

Mollusks

Within this taxonomic group, the second largest, of protostome coelomates, only 5 species have been investigated. The first account of GnRH-like immunoreactivity in a mollusk was made available in 1993, when Goldberg et al. described the distribution of GnRHir neurons in the CNS (circum-esophageal ganglia: cerebral, pleural, parietal, visceral or abdominal, and pedal) of a gastropod, the hermaphroditic pond snail *Helisoma trivolvis*. It was observed that, using two different antibodies viz. anti-mGnRH and anti-cGnRH-II, mGnRH-like immunoreactive (ir) neurons were observed in the cerebral and pedal ganglia, whereas cGnRH-II-like ir cells were observed in the parietal and visceral ganglia. No GnRHir cell bodies were described in the pleural ganglia. A differential anatomical localization of the two types of GnRH-like neurons in the mollusk has close analogy with a differential distribution of multiple forms of GnRH in neuroanatomically distinct regions in the vertebrate brain (King and Millar, 1997; Muske, 1997; Rastogi et al., 1998). In a subsequent study on *Helisoma trivolvis* and another pond snail, *Lymnaea stagnalis*, these results were confirmed with slight insignificant differences, and the distribution of GnRHir neurons in the CNS of *Lymnaea* was consistent with that in *Helisoma* (Young et al., 1999). In both studies, however, it was emphasized that the cerebral ganglia are most heavily laid with GnRHir neurons, and that the reproductive tract in both species shows GnRHir innervation. In the earlier study, furthermore, HPLC of CNS homogenate yielded multiple peaks, the major one coeluting with mGnRH (Goldberg et al., 1993). That the CNS of *Helisoma trivolvis* secretes a GnRH-like factor was further corroborated by a bioassay study in which crude CNS extract from *Helisoma* was capable of stimulating gonadotropic hormone release from dispersed goldfish pituitary cells in static incubation (Goldberg et al., 1993). Studies on GnRH-related or -like peptides in mollusks have been extended to cephalopod mollusks as well. One such study showed the localization of GnRHir perikarya in the subpedunculate lobe and the optic gland and ir fibers along the optic tract in *Octopus vulgaris* (Di Cristo et al., 1995; Di Cosmo and Di Cristo, 1998). This was later confirmed by Iwakoshi

et al. (2002). These authors cloned a precursor protein cDNA and observed that the RT-PCR transcripts were expressed in the supra- and subesophageal ganglia, optic peduncle complex, and optic gland. These observations on the presence of a GnRH-like peptide in different regions of the CNS were substantiated by enzyme-linked immunosorbent assay and TOF-mass spectrometric analysis. A dodecapeptide, with similar amino and carboxy termini as in all known vertebrate GnRH decapeptides, was characterized. Iwakoshi et al. (2002) have proposed it as octopus GnRH, and its GnRH-like activity was confirmed by a dose-dependent stimulation of LH release in primary cultures of quail anterior pituitary cells. *Loligo vulgaris*, another cephalopod mollusk, showed distinct GnRHir perikarya in the CNS (using a polyclonal anti-mGnRH, LR1, courtesy G. Benoit), including the optic gland (Di Fiore, D'Aniello A. and Rastogi, unpublished). A preliminary, three-step, purification of the optic gland homogenate (10 g sample) was performed and the extract was subjected to HPLC which revealed one major peak (retention time 36.5 min) and two minor peaks (retention times 34.5 and 45.8 min), eluting in correspondence respectively with synthetic mGnRH, cGnRH-I and sGnRH (Fig. 1). Proceeding further, a sample extract, after additional purification with Sep-Pak, was incubated overnight at 4°C together with anti-mGnRH, at a dilution of 1:25. Chromatographed under similar conditions this extract failed to show the 36.5 min major peak (Fig. 1) and thus strengthened the supposition that *Loligo* optic glands contain, at least, an mGnRH-like peptide. The presence of an mGnRH-like peptide can be suggested also in the cerebral and pedal ganglia of a bivalve mollusk (*Mytilus edulis*) in which anti-GnRH immunostains a great number of perikarya and fibers (Pazos and Mathieu, 1999).

In striking contrast with the above reports on mollusks, Zhang et al. (2000) failed to detect GnRHir elements in the CNS of an opisthobranch gastropod mollusk, *Aplysia californica*. Although no ir perikarya were observed anywhere within the CNS by using anti-mGnRH, anti-cGnRH-II, anti-IGnRH-III and anti-tGnRH-I, there was strong immunoreactivity in the connective sheath of all ganglia. RIAs also did not detect any GnRHir in the CNS. In this mollusk, a unique distribution of GnRHir material has been described: in the ovotestis, hemocytes,

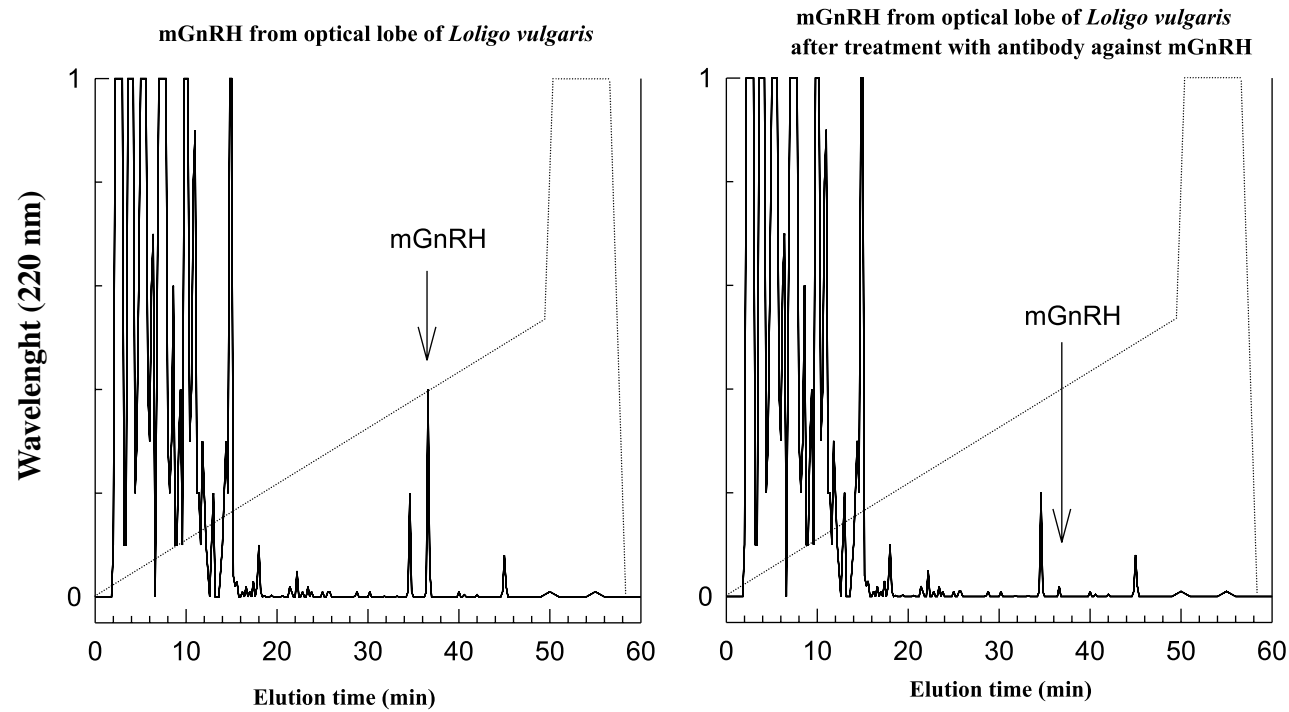


Fig. 1. High-pressure liquid chromatography elution profile of the optic lobe extract (filtered through Ultrafree Millipore-05 centrifugal filter membrane 30 kDa) from *Loligo vulgaris*, prior (left panel) and after anti-mGnRH overnight preabsorption (right panel). Arrow indicates the elution time of synthetic mGnRH, not shown in this figure.

and hemolymph (Zhang et al., 2000). Reverse-phase HPLC, coupled with specific RIAs, showed that the GnRH-like material in these sites cross-reacts with mGnRH and tGnRH-I, and that the hemolymph GnRH is biochemically and immunologically distinct from that extracted from the ovotestis and hemocytes. The timing and the width of the HPLC elution peaks indicated that *Aplysia* may possess multiple forms of GnRH-like peptides.

Arthropods

Hansen et al. (1982) described GnRHir material in the neural tissue of an insect, *Leucophaea maderae*, whereas Andriès and Tramu (1984) described it in the mesenteron of an odonate insect. Verhaert et al. (1990) also mentioned the existence of GnRHir material in the nervous system of an arthropod, the cockroach. It is puzzling that the largest animal taxon has been largely ignored about GnRH.

Echinoderms

Among echinoderms, we have detected, by immunocytochemistry, GnRHir material in the oocytes of the sea urchin, *Paracentrotus lividus*. Preabsorbed overnight with specific antigen (cGnRH-I and mGnRH), each antibody failed to immunostain oocytes any further. Gonadal extracts were purified and subjected to HPLC which allowed us to separate one major peak whose chromatographic characteristics, mass spectrometry and sequence analysis revealed that the sea urchin gonads secrete a cGnRH-I-like peptide (Rastogi, Di Fiore, Ceciliani, D'Aniello S., Branno, D'Aniello B. and D'Aniello A., unpublished). A second, minor peak elutes near mGnRH retention time, and needs further study. In vitro bioassay analysis showed that native (purified) and synthetic cGnRH-I stimulate the secretion of testosterone, estradiol and progesterone from the sea urchin gonad (unpublished).

Hemichordates

From an evolutionary point of view, hemichordates represent most probably a base group of the chordate lineage. GnRHir cells in two hemichordates, *Saccoglossus bromophenolosus* and *Ptychodera ba-*

hamensis, have been revealed by using anti-mGnRH, anti-tGnRH-I, and anti-sGnRH. These GnRHir cells are the well-known epidermal gland cells, the Mulberry cells, which are associated with neural tissue and project their basal processes into the nerve fiber layer. Thus the GnRHir Mulberry cells were found distributed widely in the ectoderm of the proboscis, collar, and anterior portion of the trunk region (Cameron et al., 1999). Ultrastructural features of the GnRHir cell types were also described and HPLC analysis, coupled with RIA, indicated the presence of a lGnRH-like molecule.

Cephalochordates

Within this chordate subgroup, there is only immunocytochemical evidence of the presence of GnRH-like peptide(s) in *Amphioxus* sp. and *Branchiostoma belcheri* (see Chang et al., 1983; Schiebman et al., 1986).

Tunicates

Of all invertebrate taxa, this group of invertebrate chordates has received the greatest attention. In fact, not only 4 different species have been investigated, but in two of them the GnRH-like peptides have been isolated, purified and sequenced. A total of four different GnRH forms are now known from this taxon: two GnRHs are vertebrate type, i.e., cGnRH-I and mGnRH, and two are unique to tunicates and were named as tGnRH-I and tGnRH-II (Powell et al., 1996; Craig et al., 1997; Di Fiore et al., 2000). To begin with, Georges and Dubois (1980) had for the first time detected the occurrence of GnRHir neurons around the cerebral ganglion and along the dorsal strand in the sea squirt, *Ciona intestinalis*. Quite some time later, a GnRH-like factor in the *Ascidella aspersa* extract was detected by RIA, and it was pointed out that it had a molecular weight little higher than that of mGnRH molecule (Dufour et al., 1988). This was soon followed by an immunological study for the detection of GnRH-like immunoreactivity in *Chelyosoma productum* (Kelsall et al., 1990). Using anti-lGnRH, immunostained material was detected in the neural ganglion and some of its roots. HPLC-RIA analysis indicated the presence of a GnRH peak strongly cross-reactive with anti-

lGnRH. A more recent immunohistochemical study, using anti-lGnRH, in this sea squirt had revealed GnRHir neurons forming a plexus around the dorsal strand and lining the wall of the blood sinus close to the gonoducts and gonads (Powell et al., 1996). GnRHir fibers also enter into the posterior nerve roots leading into the cerebral ganglion; no GnRHir cells were observed in the 'CNS'. This same study identified two novel decapeptides in this tunicate: tGnRH-I differing from mGnRH in amino acid residues 5 to 8, and tGnRH-II which differs from mGnRH and tGnRH-I not only in amino acid residues 5 to 8, but also because it is a disulfide-linked dimer (Powell et al., 1996; Craig et al., 1997). Yet in another ascidian, *Halocynthia roretzi* (Ohkuma et al., 2000), anti-mGnRH immunohistochemistry revealed its neurons in the cerebral ganglion. In *Ciona intestinalis*, further studies using either anti-cGnRH-II or anti-lGnRH confirmed the distribution pattern of GnRH-immunoreactivity on the surface area of the cerebral ganglion, along the inner wall of the dorsal blood sinus, and on the ovarian surface (anti-cGnRH-II, Mackie, 1995; anti-lGnRH, Tsutsui et al., 1998). More recently, Di Fiore et al. (2000) have isolated and purified from the *Ciona intestinalis* gonadal extract two vertebrate-type GnRHs, whose primary structure was determined as cGnRH-I and mGnRH. Anti-mGnRH and anti-cGnRH-I, both yielded a relatively strong immunoreaction in developing follicles.

Biological activities

A highly diversified neural and nonneural distribution pattern of GnRH-like material in the invertebrate groups points to the possibility that GnRH may play a variety of roles. In fact, in order to clarify the possible function(s) of GnRH in invertebrates, different synthetic GnRH forms as well as GnRH-containing tissue extract or purified native GnRH-like peptide have been used both in vivo and in vitro bioassays. The multiple functions of different forms of GnRH in a single species, among invertebrates, can be deduced partially from studies in the ascidian, *Ciona intestinalis*, which is the only invertebrate in which four forms of GnRH have been identified by determination of primary structure. In this species tGnRH-I and -II have been localized in the neural complex and along the blood sinus

and thus these forms may plausibly function as neurotransmitter or neuromodulator, whereas cGnRH-I and mGnRH produced in the gonads may function as local hormones. In vitro treatment of gonads of *Ciona intestinalis* with synthetic and native mGnRH and cGnRH-I has been shown to enhance the synthesis and release of sex steroids (Di Fiore et al., 2000). In addition to this, native mGnRH, like the synthetic molecule, induced LH release from rat pituitary fragments in vitro. Different GnRH forms (mGnRH, sGnRH, cGnRH-II, tGnRH-I, and tGnRH-II) injected into the ascidian body also have been shown to induce gamete release (probably acting as neuromodulator of other neurons innervating the gonoducts), synthetic tGnRH-I being far more effective, in a dose-dependent manner, than other GnRH forms (Terakado, 2001); in this paper, on page 282, there is the mention of tGnRH-induced spawning in yet another ascidian, *Molgula manhattensis* and on page 283 it is reported that vertebrate-type GnRHs and tGnRHs do not induce the maturation of oocytes in vitro in *Halocynthia roretzi*. In contrast with Terakado's observations, Craig et al. (1997) had observed that synthetic tGnRH-I or tGnRH-II do not induce gamete release in mature *Corella*, another ascidian genus. Both tGnRHs, nevertheless, resulted in a doubling of the gonadal content of estradiol after 24 h in *Chelyosoma productum* (see fig. 12 in Craig et al., 1997). In *Ciona intestinalis* gonads, in vitro production of estradiol and testosterone was stimulated by native and synthetic mGnRH and cGnRH-I (Di Fiore et al., 2000). A relatively similar finding was earlier reported in the amphioxus in which injection of an mGnRH agonist into the body cavity resulted in an increase in the production of estradiol and testosterone (Chang et al., 1983). It is thus clear that GnRH-like molecules do play a role in protochordate reproduction. What remains to be done is a detailed analysis of which regulatory molecule(s) controls which aspect(s) of reproduction.

Consistent with the notion of a role of GnRH in protochordate reproduction, mGnRH and cGnRH-I peptides have been observed to stimulate the sea urchin gonadal production in vitro of estradiol and testosterone (unpublished data). Whether GnRH-like peptides also play a neuromodulatory or neurotransmitter role in these deuterostomes is a theme for future research.

Among mollusks, in the water snail *Planorbis corneus*, synthetic mGnRH had been demonstrated to exert an excitatory effect on serotonergic and giant dopaminergic neurons, thus convalidating its role as a neurotransmitter or neuromodulator (Steiner and Felix, 1989). Consistent with this hypothesis, induction of electrophysiological response was observed in *Helisoma trivolvis* following injection of its nervous system extract (Goldberg et al., 1993). Data confirming a neuromodulatory role of GnRH in invertebrates are also coming from the sea pansy in which mGnRH and cGnRH-II had been observed to cause a reduction of the amplitude and frequency of rachidial peristaltic waves in a dose-dependent manner, mGnRH being 10 times more potent than cGnRH-II (Ancil, 2000). In this species, furthermore, chromatographically purified native GnRH-like peptides (fractions 1 and 2) showed a similar activity in the sea pansy peristalsis bioassay. As to the effects of GnRH-like peptides in processes related to reproduction in mollusks, Young et al. (1997) had observed that synthetic mGnRH can induce an increase in egg-laying in *Helisoma trivolvis*. GnRHir cells in ganglia innervating the reproductive system, and GnRHir innervation of the reproductive tracts in snails suggest that reproductive activities may be regulated, at least in part, by GnRH (see Young et al., 1999). Lateral lobes in the CNS of snails are the control centers of egg-laying and the presence of GnRHir cells in and around this area makes it likely that this peptide has a neuromodulatory influence on neurons of these lobes. In a recent study it was demonstrated that some vertebrate-type GnRHs exert a strong mitogenic action on dissociated gonadal cells of marine bivalves (Pazos and Mathieu, 1999). The bag cell neurons of *Aplysia* are well known as neuroendocrine neurons regulating reproductive functions and are functionally analogous to vertebrate gonadotropic cells of the pituitary; the bag cells release an egg-laying peptide hormone, in response to a characteristic electrical firing (called afterdischarge), which induces ovulation and egg-laying behavior (see Conn and Kaczmarek, 1989). In *Aplysia californica*, Zhang et al. (2000) have demonstrated that cGnRH-II can significantly decrease the duration of afterdischarge. It was suggested that GnRH may be a factor released by the ovotestis and hemocytes into the circulation to alter

neural function and the GnRH produced in hemocytes may serve as a novel mediator not only of neural functions but also of immune functions in this gastropod.

Very little is known on the regulation of GnRH synthesis and release in neural or nonneural sites in invertebrates. A recent random report in the ascidian, *Halocynthia roretzi*, which spawns at a fixed latency after sunrise, indicates that light may be involved (Ohkuma et al., 2000).

Concluding remarks

Immunocytochemical, chromatographic–radioimmunological and sequence techniques have unveiled that GnRH exists in different forms throughout the animal kingdom not only within the CNS but also outside it, in different tissues and cell types. Gonadal site of production of GnRH-like peptides is no more a prerogative of vertebrates. In fact, there is confirmatory evidence that the sea squirt and sea urchin gonads contain vertebrate-type GnRH(s).

The still valid view that GnRH acts in a diffuse manner, like a ‘neurohormone’, has made detailed anatomical studies recurrent in vertebrates. Morphological evidence strongly suggests that, for a proper understanding of GnRH’s function(s) in the nervous system and outside it, we need to know both the anatomical details of GnRH pathways and the precise location pattern. In recent years, scientific community has also switched attention to studies of GnRH receptors, cloning and sequencing of cDNAs. The presence of different forms of GnRH and their influence on different tissues in invertebrates implies the existence of multiple cognate receptor types all of which are members of the G-protein coupled receptor family (see Blomenröhr et al., 1997; Troskie et al., 1998; Safarian et al., 2001). It is conceivable that GnRH acts upon target cells through receptor-mediated process. In their paper on the effects of GnRHs on gonial DNA synthesis in a bivalve mollusk, Pazos and Mathieu (1999) have cited unpublished data, on page 117, about the occurrence of receptors for GnRH-like peptides in the gonads. The specificity of a biological action of GnRH has in many cases been ascertained by use of GnRH antagonists (see Pazos and Mathieu, 1999).

Studies involving an evolutionary approach to

GnRH have adopted one particular strategy; attention has in recent years been focused on understudied invertebrate taxonomic groups. Invertebrate species that vary in their phylogenetic relationships are likely to reveal differences (as well as similarities) in GnRH form and function. Studies on invertebrate species are beginning to provide further insights on the structural and functional similarities and differences. The perspective that evolution has acted to shape GnRH molecule effectively, leads to investigations in a comparative approach. The new explorations in the invertebrates rely on the substantial foundation provided by an enormous literature in vertebrates on the form and role of GnRH-like molecules in reproduction, although, GnRH-like peptides are also considered to act as neurotransmitter and neuromodulator (see Tsutsui et al., 1998; Zhang et al., 2000; Terakado, 2001). Molecular variation in GnRH form within a single species is also leading investigators to dissociate the specific functions of each GnRH form. In other words, multiple GnRH forms in a single species may imply multiple functions of GnRH. To sum up, the emerging evolutionary perspective of GnRH peptide(s) in vertebrates is likely to be greatly influenced by recent studies on GnRH-like peptides in the invertebrates and, as the cnidarian GnRH author (Ancil, 2000) puts it, “the evolutionary history of the GnRH family is traceable to the earliest invertebrate known to possess a nervous system” (see Fig. 2).

Whereas in tetrapod vertebrates there are only three additional forms (cGnRH-I, cGnRH-II, and guinea pig GnRH), besides the mammalian form (mGnRH), in fish groups there seems to be tendency for the ancestral decapeptide to radiate into a variety of GnRH forms, such as the lGnRH-I and II, dfGnRH, sGnRH, cfGnRH, hrGnRH, sbGnRH. In addition, some fish species do possess either the mammalian form of GnRH or cGnRH-II. This latter form was considered until recently to be the most antique form of vertebrate-type GnRH. In addition, cGnRH-I was limited to reptiles and birds. While a cGnRH-II-like form has not yet been described in an invertebrate, cGnRH-I molecular form appeared long before, in the echinoderm gonad, and successively in tunicates in which mGnRH was also isolated and characterized. It thus becomes evident that mGnRH and cGnRH-I-like molecules appeared in deuterostomes long before the onset of vertebrate lineage (Fig. 2). As of today, the primary structure of only one new form of GnRH has been revealed in a protostome invertebrate, the octopus. Although a couple of investigations have accounted for the eventual presence of a GnRH-like peptide in two different species of insects, it is surprising that arthropods have not yet been convincingly shown to possess a GnRH-like peptide. Research in more species of invertebrates would certainly yield interesting results.

Vertebrates

mGnRH, guinea pig GnRH, cGnRH-I, cGnRH-II, sGnRH, sbGnRH, dfGnRH, cfGnRH, hrGnRH, lGnRH-I, lGnRH-II

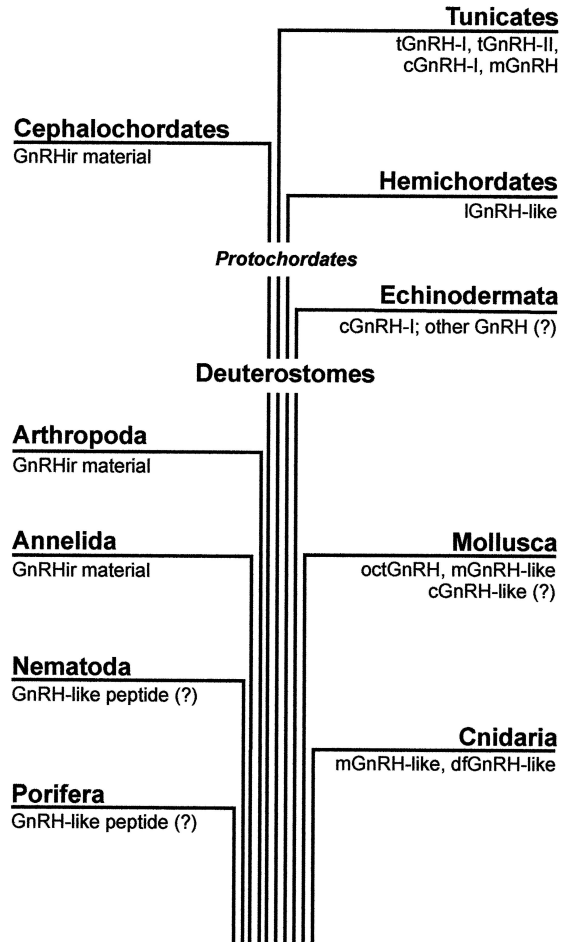


Fig. 2. Taxonomic distribution of GnRH-like peptides in invertebrates.

Acknowledgements

Our unpublished research data cited herein were financially supported by the Stazione Zoologica and the Università degli Studi di Napoli Federico II.

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CHAPTER 3

Structural and chemical guidance cues for the migration of GnRH neurons in the chick embryo

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Introduction

A body of evidence indicates that neurons producing gonadotropin-releasing hormone (GnRH) or luteinizing hormone-releasing hormone (LHRH) originate in the olfactory placode and migrate along the terminal and/or vomeronasal nerves or the olfactory nerve into the forebrain (Schwanzel-Fukuda and Pfaff, 1989; Wray et al., 1989a,b; Daikoku-Ishido et al., 1990; Muske and Moore, 1990; Ronnenkleiv and Resco, 1990; Murakami et al., 1991; Norgren and Lehman, 1991; Sullivan and Silverman, 1993; Chiba et al., 1994; D'Aniello et al., 1994; Parhar et al., 1995; Schwanzel-Fukuda et al., 1996; Amano et al., 1998). In the chick embryo, the migration of GnRH neurons from the olfactory placode starts at embryonic day (ED) 3.5. After that, GnRH neurons are generated continuously in the olfactory epithelium and migrate into the forebrain along the olfactory nerve by about ED 8. From ED 11 to the day of hatching, the majority of GnRH neurons tend to move into their adult position, whereas GnRH neurons in the olfactory epithelium have almost disappeared (Fig. 1, Murakami et al., 1991). GnRH immunoreactive (ir) terminals are detected in

the median eminence by ED 14 (Sullivan and Silverman, 1993), indicating the development of the hypothalamo–pituitary–gonadal axis.

Experimental studies with dye-labeling or chick-quail chimeric transplantation have provided direct evidence for the actual migration of GnRH neurons from the nose to the brain (Murakami and Arai, 1994a; Yamamoto et al., 1996). Labeling olfactory placodal cells with DiI resulted in sequential appearance of DiI-labeled cells expressing GnRH in the olfactory nerve, the rostral forebrain and the septo-preoptic area. Furthermore, olfactory placodectomy resulted in the absence of GnRH neurons in the nasal–forebrain axis of newts and chick embryos (Akutsu et al., 1992; Murakami et al., 1992). These results indicate that GnRH neurons arise from the epithelium of the olfactory placode.

In the central nervous system in general, neurons that are born in the ventricular layer migrate to their adult location. At least, two modes of migration are recognized in the developing brain. One mode of neuronal migration is that neuroblast migrate along the specialized glial cells known as a radial glia (Rakic, 1990). Another mode of migration is non-radial or tangential, in which neurons migrate parallel to the surface of the brain. In this type of cell migration, neuroblasts often move along the axons of preexisting neurons (Ono and Kawamura, 1989; Rakic, 1990; Kawano et al., 1995; Phelps and Vaughn, 1995) or disperse tangentially with cellular processes or with no neural structural supports (Gray et al., 1990; Ryder and Cepko, 1994; O'Rourke et

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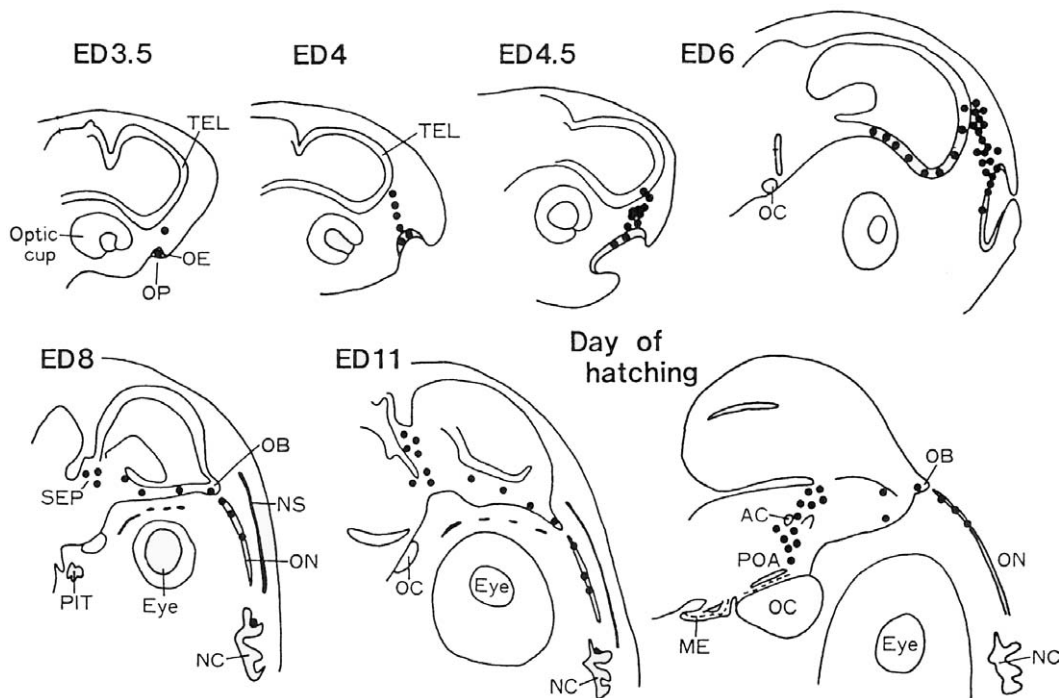


Fig. 1. Development of GnRH neuronal system in the chick embryo. AC; anterior commissure, ME; median eminence, NC; nasal cavity, NS; nasal septum, OB; olfactory bulb, OC; optic chiasm, OE; olfactory epithelium, ON; olfactory nerve, OP; olfactory placode, PIT; pituitary, POA; preoptic area, SEP; septum, TEL; telencephalon. (From Arai et al., 1997.)

al., 1995; Goleiden et al., 1997; Tamamaki et al., 1997). These gliophilic and neurophilic interaction between migrating neurons and the neural structural elements are essential for the neuronal migration (Rakic, 1990).

The migration of GnRH neurons is a unique example of neuronal migration which crosses regional boundaries within the nasal–forebrain axis. There seems some differences in mode of their migration between GnRH neurons migrating before and after the entry to the forebrain. In this article, we describe and discuss, on the basis of experimental studies, the mode of migration of GnRH neurons and the possible role of highly polysialylated NCAM (PSA-NCAM) in this process.

Migration pathway of GnRH neurons in chick embryos before the entry to the forebrain: migratory guide

The migration process of GnRH neurons from the nose to the brain is associated closely with the ol-

factory system. A variety of molecules expressed on the fibers emerging from the olfactory epithelium have been used as markers for the pathway of GnRH neurons. In chick embryos, cell adhesion molecules such as NCAM, PSA-NCAM and NgCAM delineate the pathway (Murakami et al., 1991; Norgren and Brackenbury, 1993). Transient and specific immunoreactivity to somatostatin (SST) also delineates the olfactory pathway (Murakami and Arai, 1994b). Double labeling study showed that SST-ir neural substrates are a different neuronal population from GnRH neurons. Therefore, SST is a marker for the olfactory nerve fibers in chick embryos. In rodents, NCAM, PSA-NCAM, glycoconjugates identified by CC2 antibody and the intermediate filament marker, peripherin are expressed on the axons along which GnRH neurons migrate (Schwanzel-Fukuda et al., 1992; Tobet et al., 1992; Wray et al., 1994; Yoshida et al., 1995, 1999; Toba et al., 2001).

As shown in Fig. 2, from the olfactory epithelium to the base of the forebrain, GnRH neurons course along the olfactory nerve which express PSA-

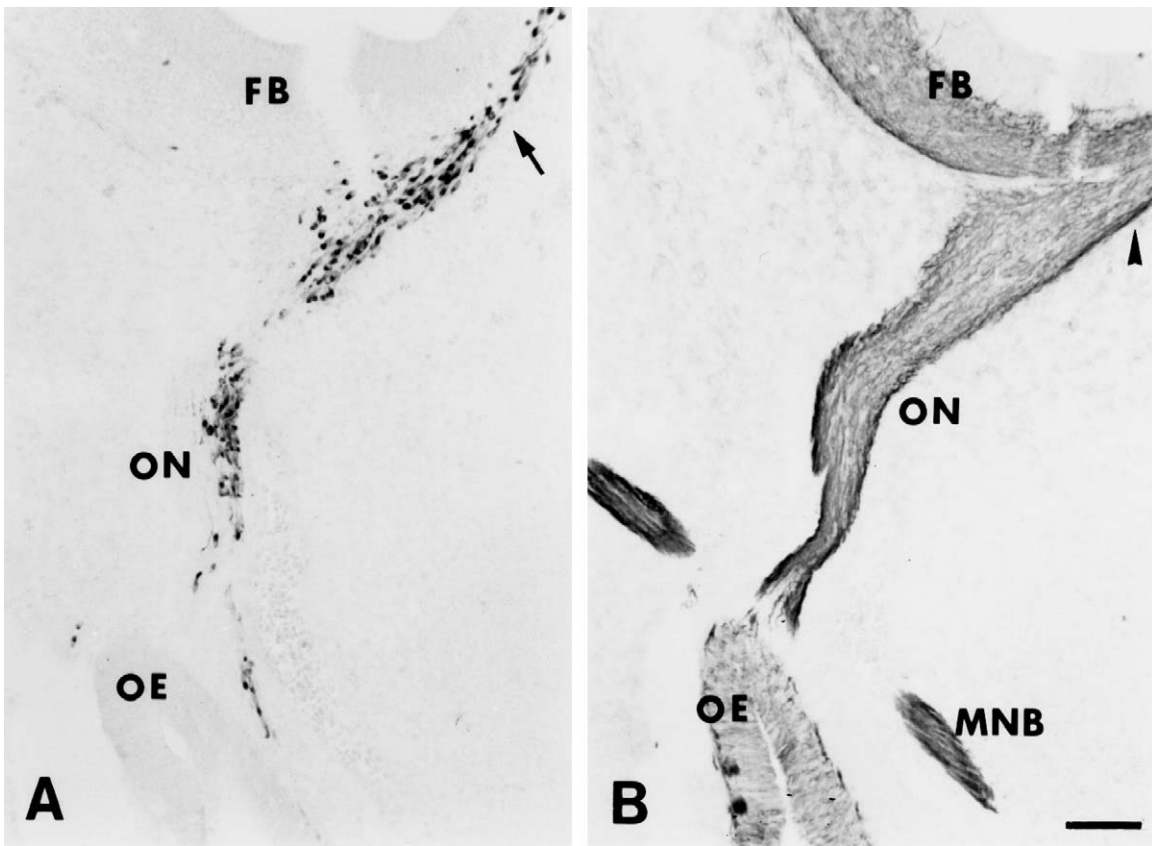


Fig. 2. Comparison of GnRH (A) and PSA-NCAM (B) immunoreactivity in the olfactory-forebrain area at ED 6.5. (A) GnRH neurons migrate along the medial portion of the olfactory nerve (ON). They enter the medial forebrain at a slightly caudal level to the junction with the olfactory nerve (arrow). (B) Section adjacent to A. A strong PSA-NCAM immunoreactivity is observed in the developing neural tissues. The olfactory nerve branches medially and its subset fibers extend to the medial forebrain (arrowhead). FB; forebrain, MNB; medial nasal branch of the ophthalmic nerve of the trigeminal nerve, OE; olfactory epithelium. Scale bar = 100 μ m.

NCAM, preferentially concentrate in the medial part of the olfactory nerve. In mammals such as rats and mice including human, the path taken by GnRH neurons is the vomeronasal and/or terminal nerves (Schwanzel-Fukuda and Pfaff, 1989; Tobet et al., 1992; Wray et al., 1994; Yoshida et al., 1995; Schwanzel-Fukuda et al., 1996). The vomeronasal organ is absent in chick (Wenzel, 1987), but it is supposed that the terminal nerve component is contained in the nasal region of adult avian species from immunohistochemistry and neural fiber tracing studies (von Bartheld et al., 1987; Wirsig-Wiechmann, 1990; Norgren et al., 1992). Although the terminal nerve cannot be discriminated from the olfactory nerve bundle in chick embryo, it is possible that the

medial part of the olfactory nerve containing GnRH neurons may correspond partly to the terminal nerve component in the chick embryo.

Migratory guide: within the forebrain

Once GnRH neurons enter the forebrain, most of them are found to be associated with caudally projecting olfactory related fibers. It is reported that these fibers express NCAM, PSA-NCAM, NgCAM, and transiently express axonal surface glycoprotein (TAG1), peripherin and SST (Schwanzel-Fukuda et al., 1992; Norgren and Brackenbury, 1993; Murakami and Arai, 1994b; Wray et al., 1994; Yoshida et al., 1995). The down-regulation of these molecules

coincides spatiotemporally with the completion of GnRH cell movement. Therefore, it has been suggested that this subset of olfactory fibers provide guiding substrate for GnRH neuronal migration within the brain (Norgren and Brackenbury, 1993; Murakami and Arai, 1994b; Wray et al., 1994). In rats, DiI labeling and immunohistochemical studies have revealed the existence of a projection that diverges from the main vomeronasal nerve, and reaches caudally into the rostral forebrain (Yoshida et al., 1995; Toba et al., 2001). The origin of caudally extending fibers is not known. Besides GnRH neurons, the olfactory placode gives rise to several different cell populations, one of which is transiently SST-expressing neurons found to migrate from the olfactory placode and located in the olfactory nerve (Murakami and Arai, 1994b). These neurons may contribute the SST-ir medial branch of the olfactory fibers that extend to the caudal forebrain.

Axon-dependent migration of GnRH neurons in the nasal region: effect of olfactory placodectomy and olfactory nerve transection

Complete ablation of the olfactory placode induces the complete loss of the nasal cavity. No GnRH neurons and fibers are detected in either the nasal region or in the forebrain on the operated side, suggesting that GnRH neurons arise from the olfactory placode (Akutsu et al., 1992; Murakami et al., 1992). The importance of the olfactory nerve as migratory guide of GnRH neurons into the forebrain has been directly shown by the olfactory nerve transection study (Murakami et al., 1998). When the pathway of the olfactory nerve is physically interrupted by a membrane filter, both the central projection of the olfactory nerve and GnRH neurons aggregate at the site of a membrane filter (Fig. 3). GnRH neurons cannot enter the brain because of the failure of the olfactory nerve targeting to the brain. Availability of a central projection of the olfactory nerve is prerequisite for the normal migration of GnRH neurons into the forebrain.

In this regard, it is of particular significance to note a report of Kallmann syndrome where GnRH neurons stay outside the brain and do not make contact with the forebrain, because of discontinuity of the olfactory-terminal nerve complex between

the nasal and the forebrain (Schwanzel-Fukuda et al., 1989). Analysis of KAL gene and its encoded protein, anosmin, has supported the possibility that genetic defect of KAL gene disrupts the terminal navigation of the early olfactory axons or olfactory bulb differentiation (Franco et al., 1991; Legouis et al., 1991, 1993; Rugarli et al., 1993; Hardelin et al., 1999).

On the other hand, in embryos with incomplete placodectomy, migrating GnRH neurons are found to deviate from their regular course along the trigeminal nerve elements (Fig. 4). This phenomenon is also observed in the case of the olfactory nerve transection in which the olfactory epithelium is almost intact (Murakami et al., 1998). Considerable number of the olfactory nerve fibers elongated along the ophthalmic nerve of the trigeminal nerve bundle in association with GnRH neurons. However, in some cases, GnRH neurons were found to migrate separately from SST-ir olfactory axons. The deviated GnRH neurons were found to migrate both the central direction and the peripheral direction of the ophthalmic nerve. In our recent findings, many GnRH neurons migrate along the spinal nerve from the olfactory placode transplanted in the forelimb region. The occurrence of GnRH neurons in anterior horn, posterior horn and spinal ganglion suggests that GnRH neurons invade the spinal cord using not only sensory axons but also motor axons as migratory guides (Murakami and Arai, 2002). These results suggest that the migration of GnRH neurons is principally axon-dependent and their identity to axonal choice for migration is not specific to the olfactory nerve in the peripheral environment.

The role of PSA-NCAM in the migration of GnRH neurons

Contact interaction between migrating neurons and structural guiding substrates plays an important role for migratory process (Rakic, 1990). It has been suggested that cell adhesion molecules (CAMs) mediate cell to cell interaction during neuronal migration (Rakic et al., 1994). The most notable molecules related to GnRH neuronal migration is neural cell adhesion molecule (NCAM). NCAM can be modified by the addition of polysialic acid (PSA). The degree of polysialylation of NCAM is regulated developmen-

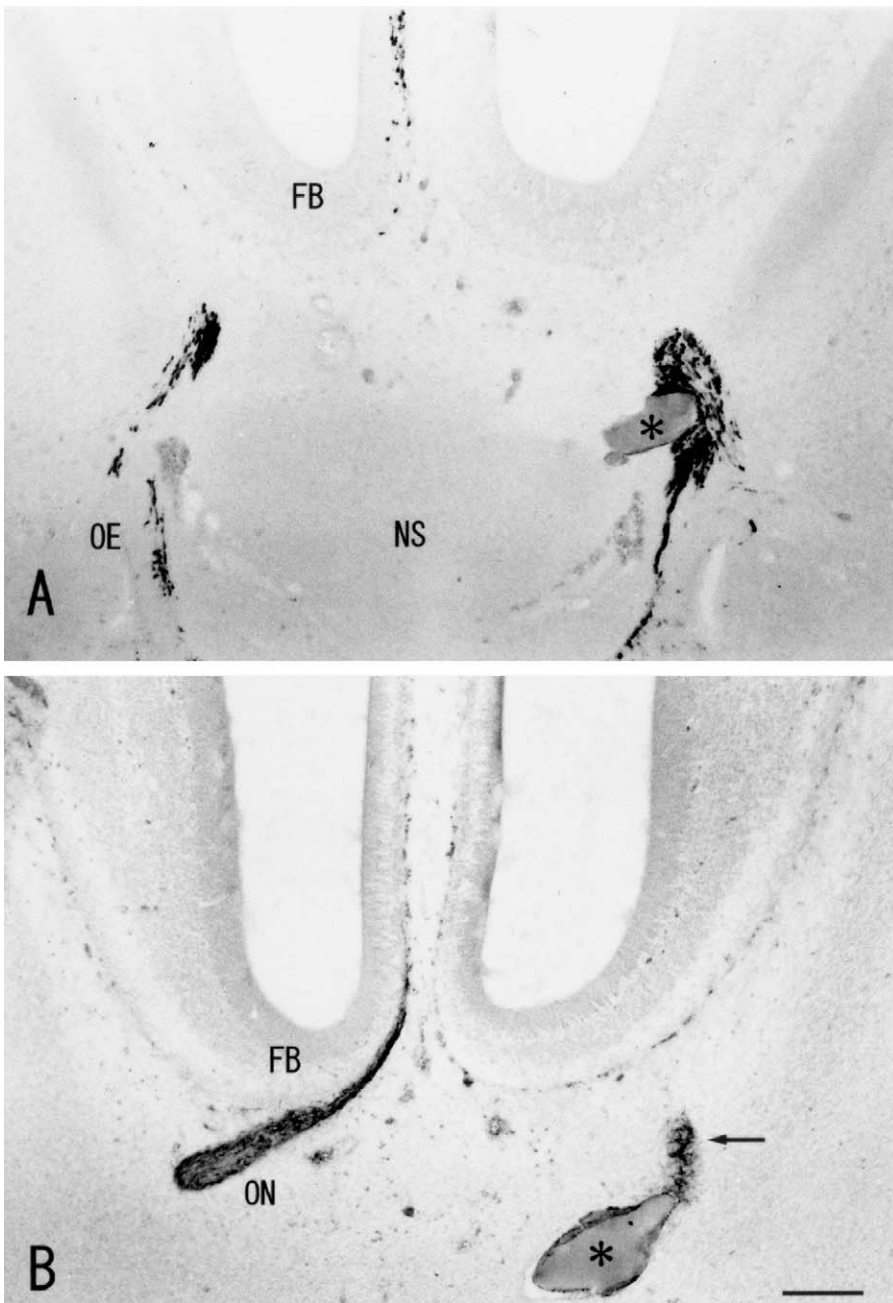


Fig. 3. The olfactory-forebrain region of an embryo treated with a membrane filter (*) sacrificed at ED 7. (A) On the operated side, GnRH-ir neurons are seen to aggregate at the site of a membrane filter (*). Note the absence of GnRH neurons in the forebrain (FB). (B) Section taken slightly dorsal level to A. SST-ir olfactory fibers cease to grow further near the membrane filter on the operated side (arrow). These remnant fibers do not contact the forebrain. NS; nasal septum, OE; olfactory epithelium. Scale bar = 250 μ m. (From Murakami et al., 1998.)

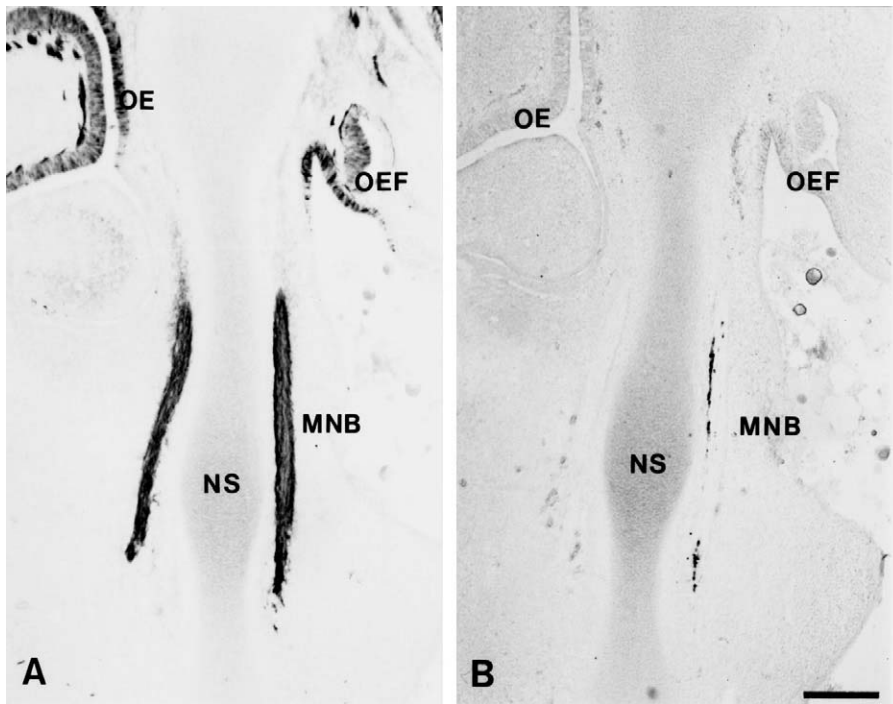


Fig. 4. The nasal region of an embryo treated with incomplete placodectomy at sacrificed at ED 7. (A) PSA-NCAM-ir fiber bundle of the ophthalmic nerve of the trigeminal nerve (MNB) are seen bilaterally. (B) GnRH-ir neurons are found migrating from the olfactory epithelium fragment (OEF) along the PSA-NCAM-ir ophthalmic nerve bundle in the peripheral direction along the nasal process. Note the absence of GnRH-ir neurons in the ophthalmic nerve on the unoperated side. NS; nasal septum, OE; olfactory epithelium. Scale bar = 250 μ m. (From Murakami et al., 1995.)

tally. PSA-NCAM has long α 2-8-linked sialic acid polymer, which have been shown to serve as an overall negative regulator of cell interactions and to be associated with cell migration and axon guidance in a number of systems during development of the nervous system (Rutishauser et al., 1988; Rutishauser and Landmesser, 1996). The developing neural tissue including the olfactory system expresses NCAM and PSA-NCAM (Seki and Arai, 1993). In chick embryos, both migrating GnRH neurons and the olfactory nerve have been found to express PSA-NCAM. Strong immunoreactivity for PSA-NCAM on GnRH neurons disappeared after these cells reached their adult positions. Therefore, it has been suggested that PSA-NCAM play an important role in the migration of GnRH neurons (Murakami et al., 1991). A close association with GnRH neurons and NCAM and/or PSA-NCAM positive olfactory/vomerolateral nerve also has been demonstrated in the olfactory placode culture system (Koide and Daikoku, 1995;

Murakami et al., 1995). Furthermore, PSA-NCAM is also positive to the trigeminal nerve into which GnRH neurons migrate when the developing olfactory nerve are physically interrupted.

To examine the role of PSA-NCAM in the migration of GnRH neurons, PSA-specific endoneuraminidase N (endoN) which selectively removes PSA from NCAM was applied in chick embryos (Murakami et al., 1998, 2000). Despite the absence of PSA, GnRH neurons were able to migrate along the olfactory nerve to arrive at the forebrain following endoN treatment. The proportion of migrating GnRH neurons in the nasal region was tend to be high in endoN treated embryos, compared to that in control embryos, suggesting that, in the absence of PSA, GnRH neurons may have some difficulty penetrating into the forebrain. However, PSA removal disrupted the normal migration pattern of GnRH neurons within the forebrain (Fig. 5). The proportion of migrating GnRH neurons in the medial forebrain

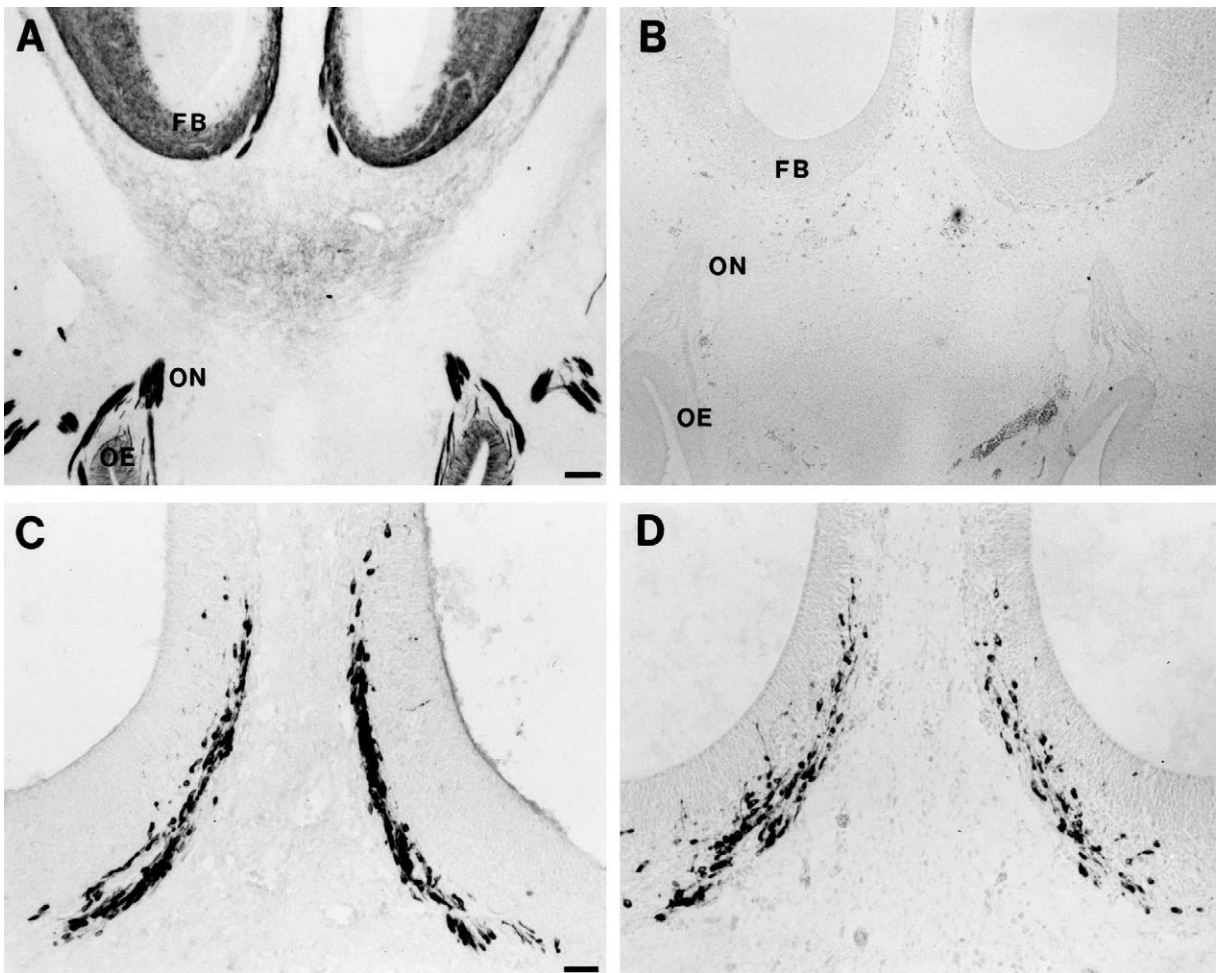


Fig. 5. Effect of PSA removal on the migration pattern of GnRH neurons in the forebrain at ED 6.5. Comparison of a control embryo (A, C) and an endoN-treated embryo (B, D). (A) Strong PSA-NCAM expression is observed in the nasal–forebrain region. (B) No PSA immunoreactivity is observed in the nasal–forebrain region after the enzymatic removal of PSA. (C) A cluster of GnRH neurons directly enter the medial forebrain and migrate along the pial surface of the medial forebrain in a control embryo. (D) After removal of PSA, the distribution of GnRH neurons in the medial forebrain surface is more dispersed compared with that of the control embryo. FB; forebrain, OE; olfactory epithelium, ON; olfactory nerve. Scale bars = 125 μ m in A; 50 μ m in C. (From Murakami et al., 2000.)

was significantly decreased in endoN-treated embryos compared to that in control embryos (Fig. 6). In mice, absence of PSA can affect the initial migration of GnRH neurons in the nasal region with a significant accumulation of these cells (Yoshida et al., 1999).

In the embryo with physical interruption of the olfactory nerve, however, enzymatic PSA removal did not only interfere with the migration of GnRH neurons into the ophthalmic nerve bundle of the trigeminal nerve, but also did not inhibit the migration of

GnRH neurons into the forebrain on the unoperated side. In mouse nasal explants, some GnRH neurons are found to migrate along the NCAM-negative olfactory axons (Wray et al., 1994). Outside the central nervous system, other molecules appear to play a role in GnRH cell movement along the nerve fibers. For example, cell surface-associated extracellular matrix such as phosphacan is found in the GnRH neurons and the olfactory nerve in chick embryos (Nishizuka et al., 1996). Since phosphacan is known to bind NCAM and NgCAM (Grumet, 1990), heterophilic

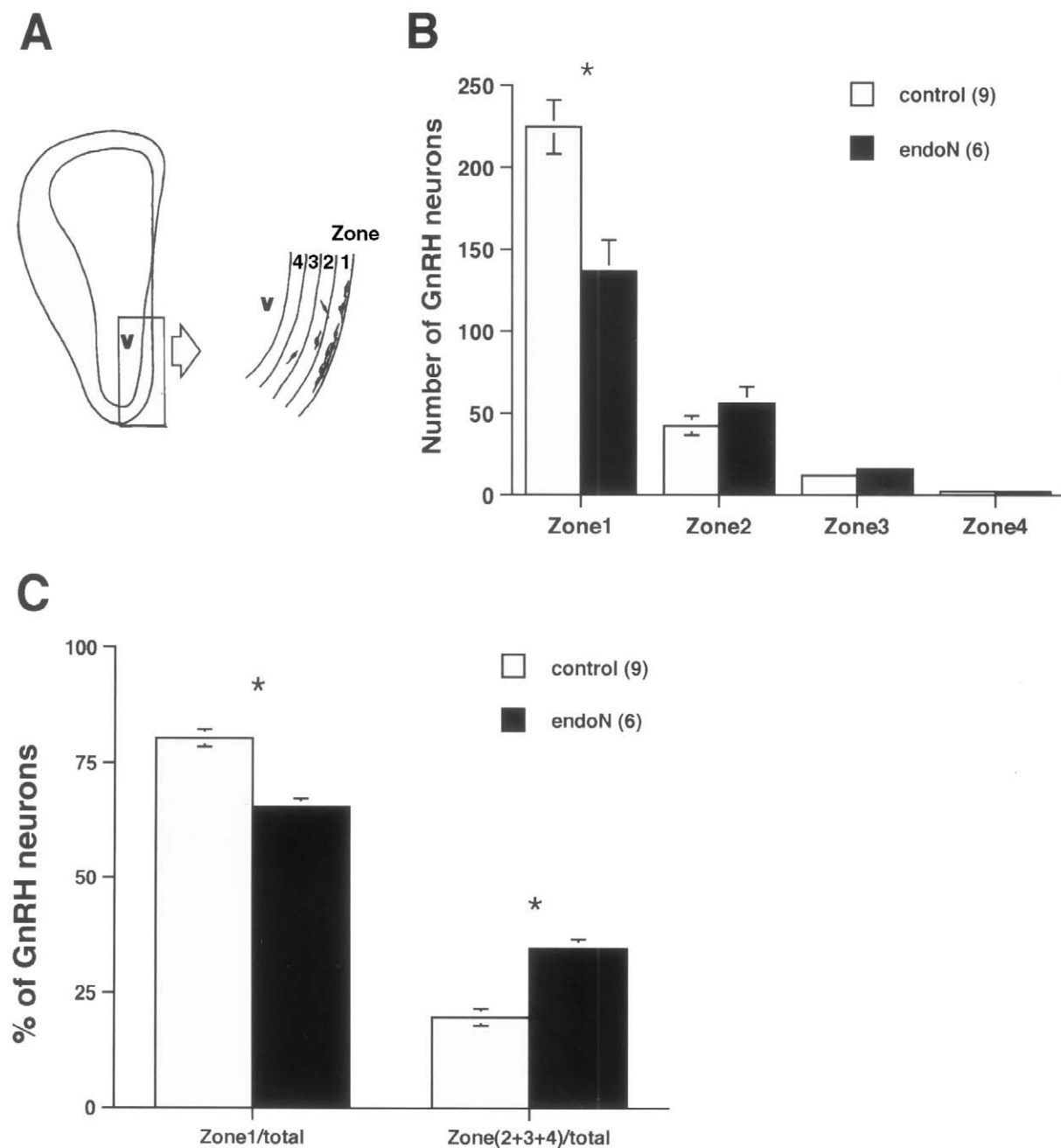


Fig. 6. Quantitation of the effect of PSA removal on the distribution of GnRH neurons in the medial forebrain at ED 6.5. (A) relative distribution of GnRH neurons was measured by subdividing the medial forebrain into four zones (1–4) parallel to the brain surface. (B) Histograms of the mean GnRH neurons number in each zone. Although most GnRH neurons are located in zone 1 for both endoN-treated embryos and control embryos, endoN treatment resulted in a significant reduction in the number of GnRH neurons in this region (asterisk, $P < 0.004$). (C) The percentage of total GnRH neurons in zone 1 and zones 2–4. Note that, after PSA removal, the relative location of GnRH neurons shifts significantly from zone 1 to zones 2–4 (asterisks; $P < 0.0001$). V, lateral ventricle. (From Murakami et al., 2000.)

binding with NCAM between GnRH neurons and the olfactory axons may be involved in the migration of GnRH neurons.

Development of migration pathway within the forebrain

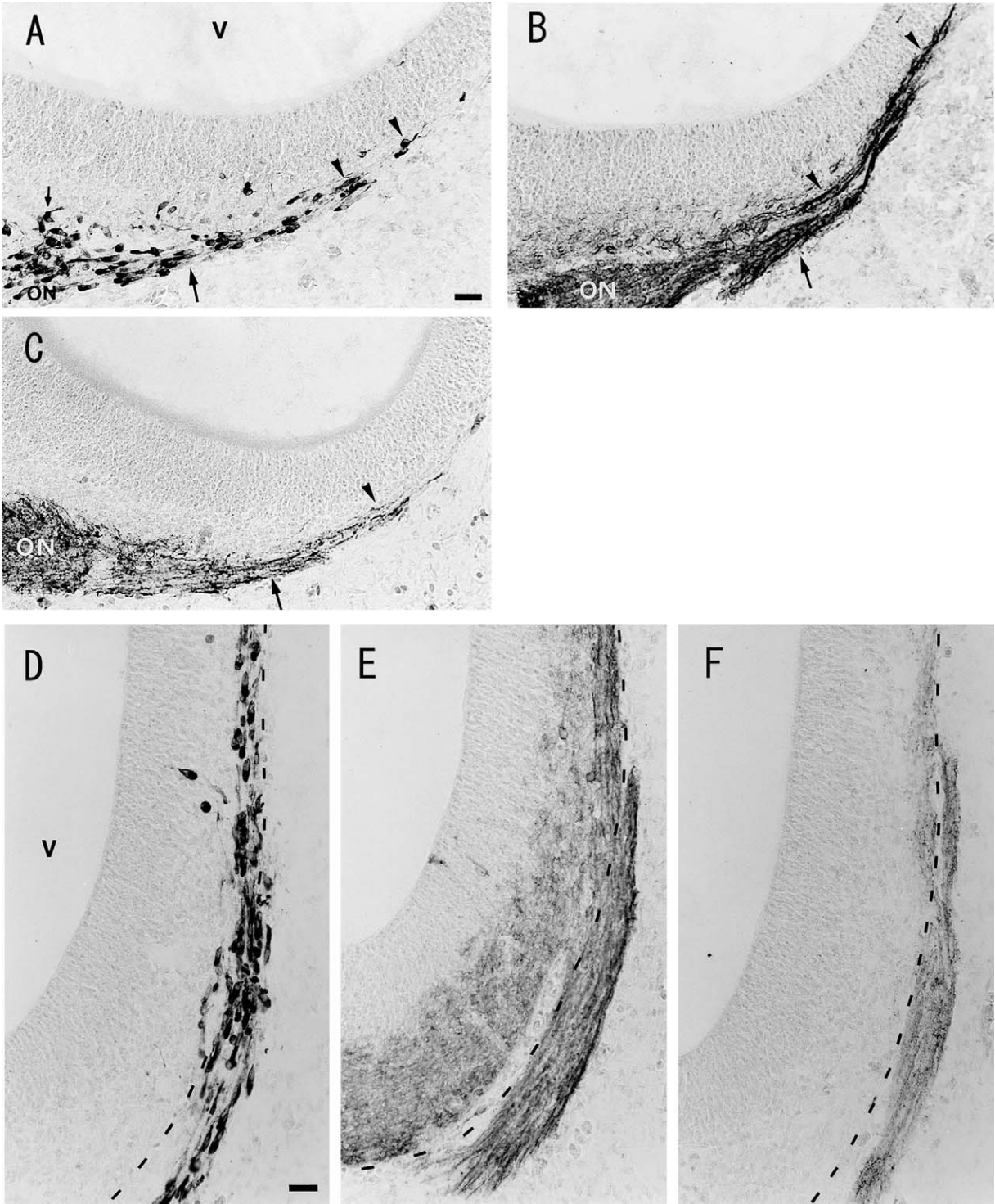
The exact pathway of GnRH neurons to their final location within the brain is unclear to date. We observed the progressive distribution changes of GnRH neurons with the extension of a subset of olfactory fibers, which is specifically immunoreactive to SST in chick embryos (Murakami et al., 2000). At ED 4.5, a few pioneer GnRH neurons enter the rostral forebrain where the olfactory nerve contacts with the presumptive olfactory bulb. No detectable guiding structural substrates for their migration are found in the rostral forebrain at that stage. A small number of GnRH neurons continue to enter the brain at the junction with the olfactory nerve, and they disperse into the presumptive olfactory bulb and in the direction caudal to the medial forebrain. These GnRH neurons appear to migrate in no association with any neural structures by ED 5, since a well defined migration pathway of GnRH neurons in the brain is not yet recognized. Considerable change occurs at ED 5.5–6. One component of the olfactory nerve branches to form a small bundle of axons before entry to the forebrain. These fibers can be identified by SST and PSA-NCAM antibodies as a subset of the olfactory nerve and extend toward the ventromedial forebrain (Fig. 7B, C). These fibers then invade directly the medial forebrain, accompanying with many GnRH neurons (Fig. 7A). By ED 6.5, migration pathway of GnRH neurons in the brain becomes easily recognizable (Fig. 7D–F). Large numbers of GnRH neurons in small clusters entered the medial forebrain and then migrated with the medial branch of the olfactory nerve. Most of these GnRH neurons migrate along the brain surface beneath the pia mater. The migration direction is primarily tangential.

During ED 6.5–7.5, this pathway extends near the preseptal area. The course of medial branch of the olfactory nerve is also specifically identified by antibody to axonin-1, which is the chick homolog of TAG-1 and can promote neurite outgrowth in vitro (Stoeckli et al., 1991; Hasler et al., 1993). Axonin-1-ir fibers seem to be identical to a transient expression

of TAG-1 on the caudal branches of the vomeronasal nerve in rats (Yoshida et al., 1995). In our materials, axonin-1-ir fibers extended to the parenchyma near the roof of the third ventricle, where many GnRH neurons were apart from these fibers and turn ventrally toward the preoptic area (Fig. 8A–C; unpublished data). At ED 11, GnRH neurons almost reached their adult positions, whereas SST and axonin-1 immunoreactivity on the medial branch of the olfactory nerve disappeared.

On the basis of these observations, it is proposed that at least two major pathways of GnRH neuronal migration exist in the brain. The main pathway is characterized by a small cluster of GnRH neurons that are aligned continuously along the medial forebrain surface dorsocaudally. It has been implicated that axophilic migration of GnRH neurons occurs in this pathway that largely corresponds to the course of a subset of the olfactory fibers. The migration of GnRH neurons on this pathway appears to be regulated by PSA-NCAM. PSA removal disrupted the specific pattern of GnRH neurons within the medial forebrain. Significant declustering of GnRH neurons and deviation of migrating GnRH neurons from the main pathway occurred (Figs. 5 and 6). Because the axonal pattern of the medial branch of the olfactory nerve, migration substrate within the brain was not affected, a defect on the GnRH neurons could not be a secondary effect.

Another pathway is independent of the medial branch of the olfactory nerve. For example, before these fibers invade the forebrain, a small number of GnRH neurons enter the forebrain and disperse in the presumptive olfactory bulb and the medial forebrain. After a subset of olfactory fibers entered the medial forebrain in association with GnRH neurons, some GnRH neurons still disperse at multiple levels in the main pathway, especially, the diverse dispersion of GnRH neurons occurs at the most caudal level of the main pathway. In this case, many GnRH neurons seem to leave the main pathway and to change their course in the direction of the ventral preoptic area. However, it might be possible that the pioneer GnRH neurons that entered the forebrain in the earlier stage join a group of these deviated GnRH neurons. In either cases, it remains unclear the structural and chemical guiding substrates for the migratory routes of these GnRH neurons.



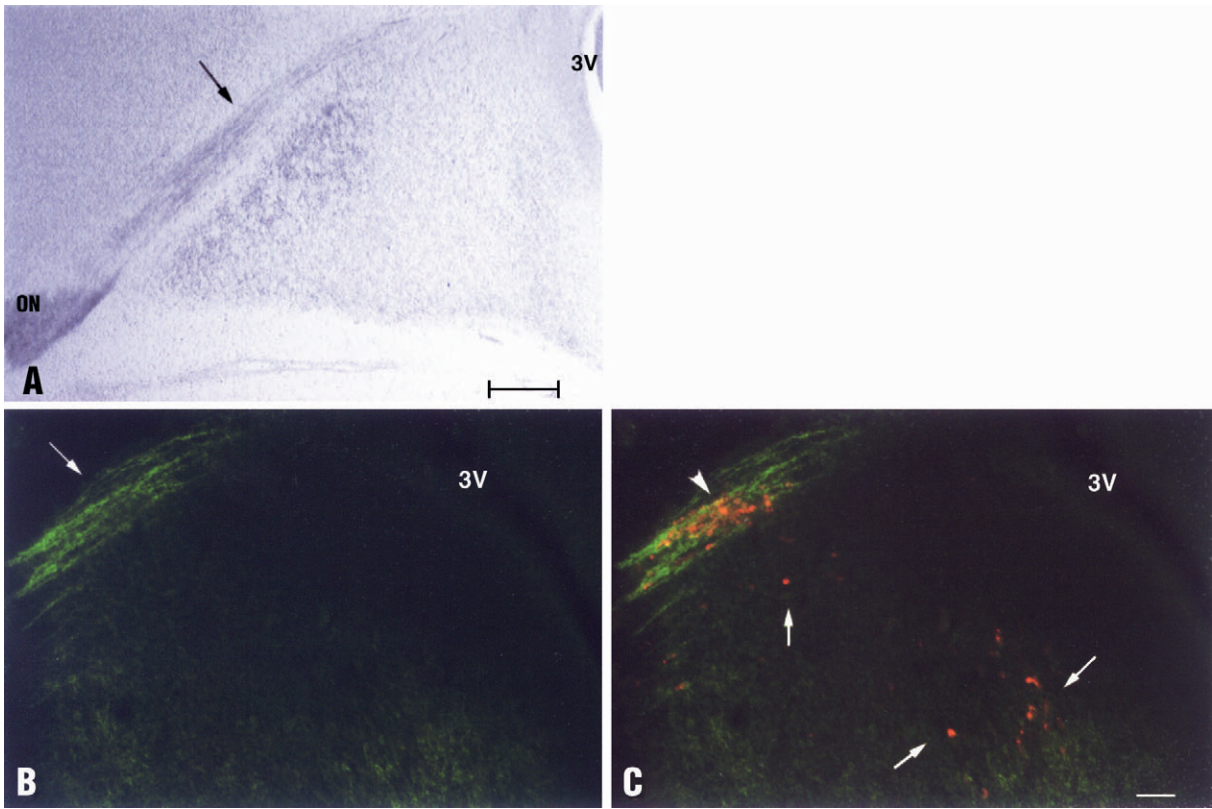


Fig. 8. Sagittal sections through the forebrain at ED 7 embryos. Axonin-1 immunostaining (A) and double fluorescence for GnRH and axonin-1 (B, C). Rostral is to the left. A: A subset of olfactory fibers that is immunoreactive to axonin-1 branches from the olfactory nerve (ON) and extend into the forebrain dorsocaudally (arrow). (B) Axonin-1-ir fibers (arrow) are seen to extend along the medial forebrain surface beneath the pia mater, and appear to terminate the parenchyma near the roof of the third ventricle (3V). (C) The merged image of the same section as in B, with axonin-1 (green) and GnRH (red). Many GnRH neurons are seen to associate with axonin-1-ir fibers (arrowhead). At the most caudal level of the course of axonin-1-ir fibers, some LHRH neurons are seen to scatter in direction to the ventral forebrain (arrows). These GnRH neurons seem to deviate from the main pathway and turn to the ventral area. Note the absence of axonin-1-ir fibers in the ventral region where GnRH neurons disperse. Scale bars = 200 μm in A; 30 μm in B.

Conclusion

In the nasal region, evidence indicates that the olfactory nerve guides the migration of GnRH neurons

into the forebrain during normal development. However, a straying phenomenon of GnRH neurons into the ophthalmic nerve of the trigeminal nerve suggests that GnRH neurons could use any type of

Fig. 7. Coronal sections of the rostral forebrain at ED 5.5 (A–C) and ED 6.5 (D–F). (A) Many GnRH-ir neurons migrate in direction to the medial forebrain (large arrow) and enter the ventromedial forebrain. These cells migrate along the region close to the pial surface of the brain (arrowheads). A few GnRH-ir cells enter the brain at the junction of the olfactory nerve (ON, small arrows). (B) Section adjacent to A. PSA-ir fibers that are branching medially from the main olfactory nerve bundle extend toward the ventromedial forebrain (arrow). A portion of PSA-ir fibers enter the medial forebrain and extend along the pial surface (arrowhead). (C) Section adjacent to B. The SST-ir fibers branch medially from the olfactory nerve and extend toward the ventromedial forebrain (arrow), accompanying by migrating GnRH neurons. A subset of fibers enters the forebrain (arrowhead). (D) A small cluster of GnRH-ir cells directly enter the medial forebrain and course along the medial forebrain surface. (E) Section adjacent to D. PSA-ir fibers branch from the olfactory nerve accompanied by PSA-positive GnRH-ir cells and enter the forebrain. (F) Section adjacent E. SST-ir fibers branch from the olfactory nerve and extend along the migration pathway of GnRH neurons. The dashed line in D–F indicates the pial surface. V; lateral ventricle. Scale bars = 30 μm in A; 25 μm in D. (From Murakami et al., 2000.)

axons as guidance substrate for their migration. This conjecture is well reinforced by the fact that GnRH neurons were detectable in the spinal nerve and also in the spinal cord following placode graft transplantation in the forelimb region. The presence of structural cues seems to be of importance primarily in the migration of GnRH neurons in the peripheral region.

Within the forebrain, examination of the normal development of GnRH neurons indicates that the main pathway of GnRH neuronal migration largely corresponds to the course of a subset of the olfactory fibers, suggesting a kind of axon-dependent migration of these GnRH neurons. PSA-NCAM is more likely to regulate the interaction of the migrating neurons with their axonal substrates in the brain, whereas it may not be important for the migration of GnRH neurons along the peripheral neural elements. Another migration mode taken by GnRH neurons is independent of the olfactory related fibers. The substrates and chemical cues for their migration remain largely unknown. Although the final migration pathway of GnRH neurons to their adult positions is not yet clear, the dispersion of GnRH neurons from the main pathway (Fig. 8B, C) implicates that GnRH neurons may show a final phase of the migration targeting on the preoptic-hypothalamic area. A subset of the olfactory fibers as guiding substrate may act as a kind of temporary scaffolding for GnRH neurons to move to the subsequent pathways that lead to the final site. More detail analysis of the migration pathway of GnRH neurons to their permanent location is needed for the understanding the structural and molecular substrata that guide them.

Acknowledgements

We would like to thank Dr. K. Wakabayashi for generous supply of LHRH monoclonal antibody, LRH13. This study was supported by Grants-in Aid from the Ministry of Education, Science and Culture of Japan and by Japan Private School Promotion Foundation.

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CHAPTER 4

What defines the nervus terminalis? Neurochemical, developmental, and anatomical criteria

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Introduction

The nervus terminalis — a neuromodulatory system

The *nervus terminalis*, or terminal nerve, is a diffusely organized system of neurons that lie within the nasal cavity and rostral forebrain of all jawed vertebrates, including humans (Fig. 1). Its most significant feature is that some of its neurons contain the reproductive neuropeptide, gonadotropin-releasing hormone (GnRH). External to the forebrain, the cell bodies of the nervus terminalis are typically embedded within chemosensory nerves in the nasal cavity or they may be congregated in compact ganglia, commonly in the region of the olfactory bulbs. The neural processes of the plexus project to peripheral sensory structures (e.g. olfactory and/or vomeronasal mucosa, Wirsig-Wiechmann, 1993; Wirsig-Wiechmann and Wiechmann, 2001; retina in fish, Stell et al., 1984; Oka et al., 1986) and to multiple areas of the brain (Oka, 1992). While there is some variation in the extent and complexity of the projections among species, the projections

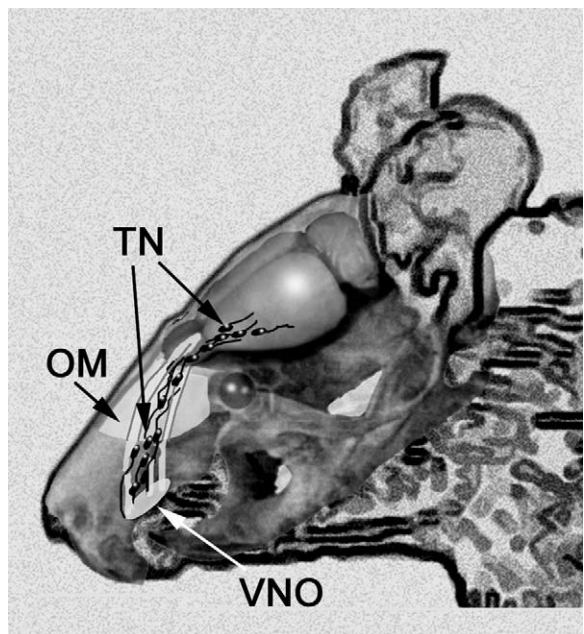


Fig. 1. Diagram of a rodent head showing the location of the *nervus terminalis* (TN, black bipolar cells) in relation to the brain and nasal cavity. In rodents, these TN neurons are embedded within vomeronasal nerves on the nasal septum and along the rostromedial surface of the olfactory bulbs. More caudally the plexus separates from the vomeronasal nerve and lies along the caudoventral surface of the olfactory bulb before penetrating the brain substance in the region of the anterior olfactory nucleus. OM, olfactory mucosa; TN, *nervus terminalis*; VNO, vomeronasal organ.

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to peripheral olfactory receptive regions and forebrain are consistently present in all species studied. Also consistent is the presence of GnRH in some or all neurons within the nervus terminalis complex (Schwanzel-Fukuda and Silverman, 1980).

History: the discovery of the nervus terminalis

The nervus terminalis was first described as an 'überzahliger Nerv' (supernumerary nerve) distinct from the olfactory system in *Galeus canis*, the smooth dogfish, by Fritsch (1878). This discovery was made possible by the fact that the nervus terminalis system in sharks is anatomically separate from the olfactory tracts, i.e. it can be visually identified in gross dissected preparations of selachian rostral cranium as a separate ganglionated nerve bundle lying along the olfactory tract (Fig. 2). This initial discovery was followed by numerous gross and microscopic examinations of the nervus terminalis neural plexus in various vertebrate species. In most of these studies nonspecific labeling techniques (e.g. Nissl, Golgi, pyridine silver) were used that allowed a somewhat incomplete determination of

the location of cell bodies and axonal projections within peripheral olfactory structures and forebrain. In 1980, Schwanzel-Fukuda and Silverman reported that a population of neurons within the nervus terminalis system contains GnRH. This discovery made it possible to study the distribution and projections of nervus terminalis neurons in a much more detailed manner. It also led to the speculation that the nervus terminalis is involved in some aspect of reproduction.

Recent studies have strongly suggested that the GnRH-containing neurons of the nervus terminalis are neuromodulatory to peripheral chemosensory neurons (Eisthen et al., 2000), to retina (Stell et al., 1984) and to brain (Oka and Matsushima, 1993). It is likely that this neuromodulatory system is important for reproduction. Gonadotropin-releasing hormone is known to facilitate sexual behaviors when GnRH receptors are stimulated in specific forebrain and midbrain areas (Moss and McCann, 1973; Moss and Foreman, 1976; Moore et al., 1982). The concentration of GnRH in the nervus terminalis has been shown to fluctuate during courtship and mating behavior (Propper and Moore, 1991) suggesting that the nervus terminalis is activated during sensory-induced reproductive behaviors. Likewise, lesions of the nervus terminalis alter reproductive behaviors (Wirsig and Leonard, 1987; Wirsig-Wiechmann, 1997; Yamamoto et al., 1997).

The GnRH neurons of the nervus terminalis form a system that appears to be functionally separate from the preoptic GnRH neurons in most animals, i.e. the nervus terminalis is generally not involved with gonadotropin release. This is supported by the fact that in many species there are separate forms of GnRH (Lovejoy et al., 1992; Parhar et al., 1998b), separate precursor cells (Whitlock et al., 2000) and, in some cases, differences in the timing of initial GnRH synthesis by the two populations of neurons (Parhar, 1997). However, the common migratory route from the olfactory placodal region into the brain of the nervus terminalis and preoptic/hypothalamic group of GnRH neurons (see below) suggests a close relationship between these two systems. Thus, the nervus terminalis system may represent a coordinating system for reproductive behavior and physiology that may act in concert with the preoptic/hypothalamic GnRH system.

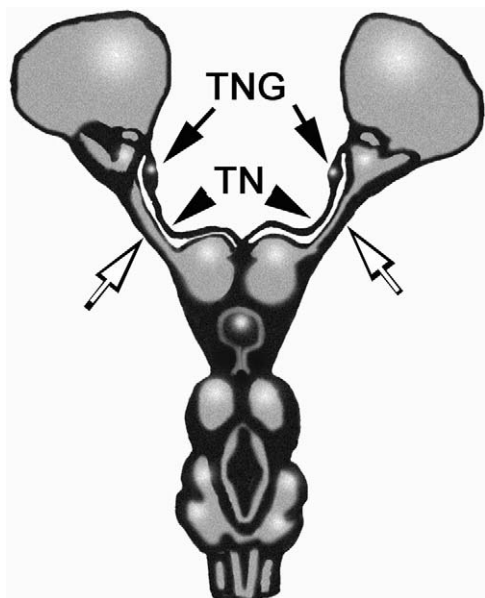


Fig. 2. Diagram of the dorsal aspect of a shark brain demonstrating the location of the nervus terminalis (TN) nerve and ganglion (TNG). In sharks, the nervus terminalis is physically separated from the olfactory tracts (open arrows).

While a substantial amount of information has been gathered over the past 20 years on the GnRH component of the nervus terminalis, there has been no formal analysis of which specific and consistent features can be used to define the nervus terminalis system as a functional unit. The purpose of this review is to propose: (1) developmental, (2) neurochemical, and (3) anatomical features that combined can be used to define the nervus terminalis in all species. Interspecies differences that may signify evolutionary trends will also be discussed.

Development of the nervus terminalis and preoptic/hypothalamic GnRH system

The GnRH neurons that come to lie along the nervus terminalis in the nasal cavity and rostral forebrain as well as those that reside in the preoptic/hypothalamic areas migrate out of the olfactory placodal region during development (Schwanzel-Fukuda and Pfaff, 1989; Wray et al., 1989). These neurons migrate along a path on the nasal septum where the mature nervus terminalis will eventually lie (Schwanzel-Fukuda et al., 1981; Schwanzel-Fukuda and Pfaff, 1990). Some GnRH neurons of the nervus terminalis migrate into the ventral forebrain but many remain in the nasal cavity and in peripheral autonomic ganglia associated with the nasal cavity (Wirsig-Wiechmann and Lepri, 1991; Wirsig-Wiechmann, 1993). There is no evidence that any nervus terminalis cells arise from the neural tube.

Numerous studies have demonstrated that removal of the olfactory placode at certain stages of development eliminates both nervus terminalis and preoptic/hypothalamic GnRH systems (Akutsu et al., 1992; Murakami et al., 1992; Northcutt and Muske, 1994). While it was originally thought that GnRH neurons originated from the same precursor group as olfactory neurons, there is now evidence that GnRH neurons arise from neural crest cells that migrate very early into the region of the olfactory placode (Whitlock et al., 2000). The groups of GnRH neurons destined for the nervus terminalis and preoptic/hypothalamic groups, respectively, arise from separate areas of the neural plate before their migration into the placode.

Other neurochemically separate components of the nervus terminalis (see below) also arise from the

olfactory placodal region, (e.g. molluscan cardioexcitatory peptide/neuropeptide Y (FMRFamide/ NPY)-like immunoreactive neurons, Northcutt and Muske, 1994; Hilal et al., 1996). In addition to the nervus terminalis and preoptic GnRH neurons, it appears that the olfactory placode gives rise to groups of neurons that migrate even further into the brain, such as tyrosine hydroxylase-immunoreactive neurons (Verney et al., 1996). The fate of these latter neurons is not known. Whether these neurons are functionally connected with the nervus terminalis system is also not known.

Based on this developmental information, we propose that one of the defining characteristics of the nervus terminalis is that its neurons arise from the neural crest region of the neural plate, move into the region of the olfactory placode and migrate from there into peripheral nasal areas and ventral forebrain areas.

Neurochemical components of the nervus terminalis

Multiple forms of GnRH are present in vertebrate brains. Typically there are at least two forms of GnRH: a varying form (e.g. chicken-I GnRH, King and Millar, 1982a,b; salmon GnRH, Sherwood et al., 1983) within the forebrain and a 'chicken-II' form most commonly found within the midbrain (Miyamoto et al., 1984). In addition, there can be neurochemically dissimilar forms of GnRH between the nervus terminalis and preoptic/hypothalamic neural groups (Parhar et al., 1998b; Chiba et al., 1999) that are differentially regulated. This supports the hypothesis that the nervus terminalis and preoptic/hypothalamic neurons are functionally separate groups (Soga et al., 1998). In addition, the nervus terminalis itself may contain multiple forms of GnRH (Lovejoy et al., 1992; Forlano et al., 2000).

Besides GnRH, other neuropeptides, neurotransmitters or associated enzymes are co-localized in GnRH neurons or found in a separate population of neurons within the nervus terminalis plexus. Compounds that have been consistently identified in nervus terminalis across multiple classes of animals are (1) a neuropeptide tyrosine (NPY)-like peptide (that can also be visualized by antibodies against molluscan cardioexcitatory peptide, FMRF-

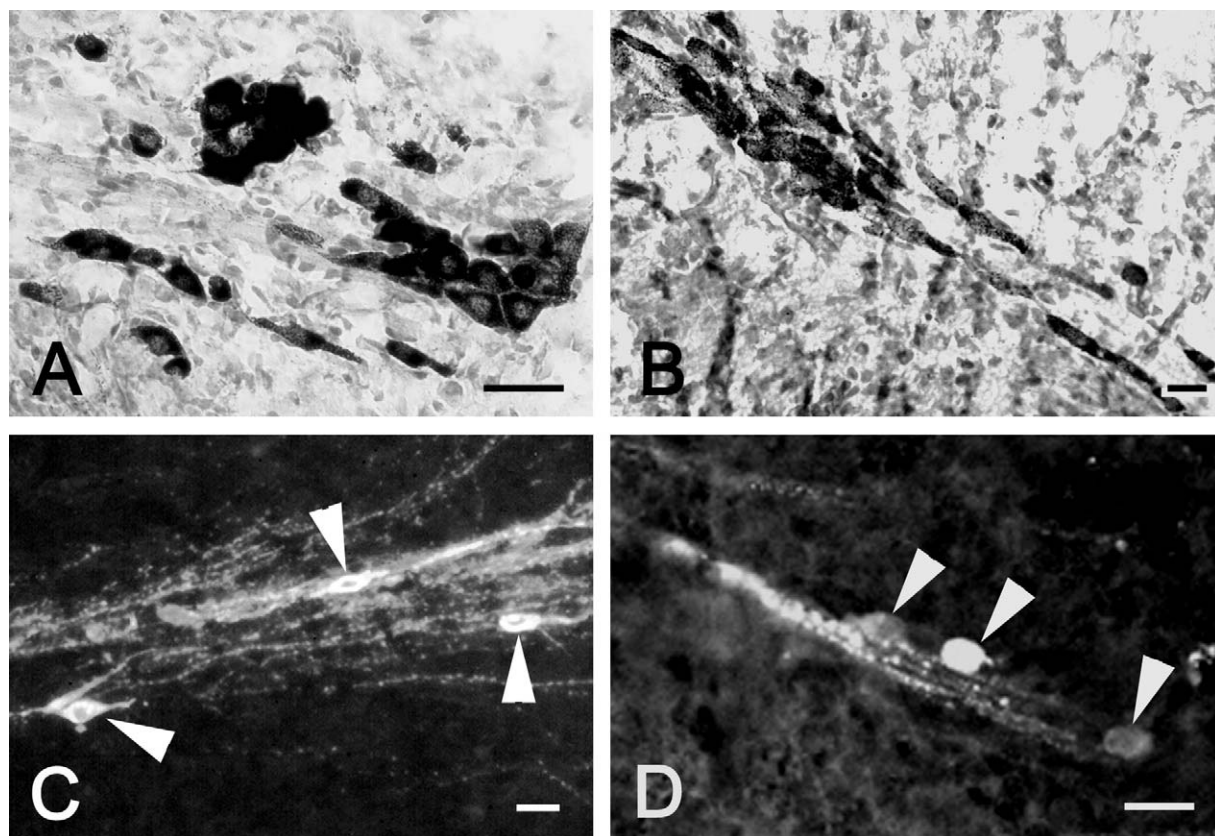


Fig. 3. Micrograph of nervus terminalis neurons on the medial surface of the olfactory bulbs of hamster labeled with (A, B) acetylcholinesterase histochemistry and (C, D) GnRH immunocytochemistry. (A) These neurons can be grouped into tight clusters, or (B) distributed along the nervus terminalis nerve fibers. (C, D) There are fewer GnRH containing neurons (arrowheads) along the nervus terminalis. Bars = 20 μ m.

amide; Stell et al., 1984; Muske and Moore, 1988; Wirsig-Wiechmann, 1990; Oelschlager et al., 1998; D'Aniello et al., 2001) and (2) cholinergic related enzymes (choline acetyltransferase, Schwanzel-Fukuda et al., 1986; and acetylcholinesterase, Wirsig and Getchell, 1986; Wirsig and Leonard, 1986; Wirsig-Wiechmann, 1990; White and Meredith, 1995). Most frequently, GnRH and the NPY-like peptide are present in separate populations of nervus terminalis neurons (White and Meredith, 1995), but these peptides can be co-localized (Stell et al., 1984; Wirsig-Wiechmann and Oka, 2002). The presence of cholinergic enzymes suggests that neurons along the nervus terminalis also contain acetylcholine. White and Meredith (1993, 1995) have supplied further evidence for a cholinergic component. They

have shown that nervus terminalis ganglion neurons in sharks are responsive to acetylcholine. Heavy acetylcholinesterase labeling, which is suggestive of cholinergic function rather than cholinceptive function (receiving cholinergic input), usually occurs in the population of nervus terminalis neurons that does not contain GnRH (Wirsig and Leonard, 1986; White and Meredith, 1995). These potential cholinergic neurons seem to be much more numerous than GnRH neurons along the nervus terminalis of rodents (Wirsig and Leonard, 1986; Fig. 3). Therefore, in many species, it is most likely that acetylcholine and the NPY-like peptide are commonly present in the same group of neurons and that the GnRH neurons represent a separate population.

There have also been reports of the presence of vasoactive intestinal peptide (Schwanzel-Fukuda et al., 1986) and glutamate (Yamamoto et al., 2001) in nervus terminalis. As will be discussed below, the presence of compounds in addition to GnRH in neurons of the nervus terminalis varies among species. For example, most non-mammalian species appear to possess an NPY-like neural component of the nervus terminalis, but few mammalian species have demonstrated such a component (Oelschlager et al., 1998). This may represent the unavailability of antisera to label the mammalian homologue of the NPY-like compound found in fish, reptiles, amphibians and birds.

Based on this neurochemical information, we propose that a second defining characteristic of the nervus terminalis is that at least one population of its neurons contains a form of GnRH, and another or same population of neurons contains acetylcholine and/or an NPY-like compound.

Neuroanatomical characteristics of the nervus terminalis

Nervus terminalis cell body location and characteristics

The location and distribution of nervus terminalis neural cell bodies differs between species. However, in most species, the neural cell bodies are diffusely distributed along the fibers of the nervus terminalis neural plexus both within the nasal cavity and within the forebrain (see Fig. 1; Wirsig and Getchell, 1986; Wirsig and Leonard, 1986; Wirsig-Wiechmann, 1993). In addition, the entire plexus is embedded within other nerve fascicles in the nasal cavity. In a few species (sharks and teleosts), the neurons are packed into a ganglion located external to (see Fig. 2; Fritsch, 1878; White and Meredith, 1995) or internal to the forebrain at the junction between the olfactory bulb and telencephalon (Grober et al., 1987; Oka, 1992). In mammals (Wirsig-Wiechmann and Lepri, 1991) and amphibians (Wirsig-Wiechmann, 1993; Wirsig-Wiechmann and Ebadifar, 2002), GnRH neurons have also been found in the pterygopalatine and palatine ganglia, respectively (Fig. 4). Most recently we have also observed the presence of FMRFamide/NPY-like neu-

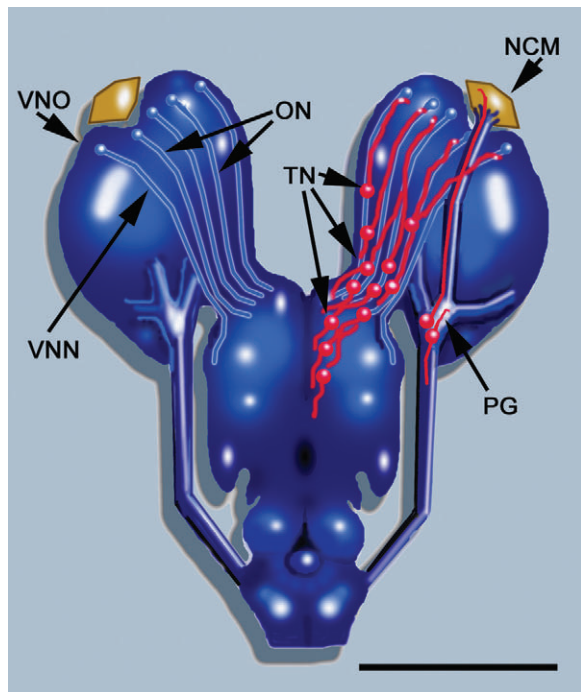


Fig. 4. Diagram of a ventral view of tiger salamander brain and nasal cavity illustrating the location of GnRH nervus terminalis neurons (TN, red neurons) in the olfactory and palatine neural systems. GnRH neurons of the palatine system project to the naris constrictor muscle (NCM) located between the vomeronasal organ (VNO) and rostral tip of the main olfactory chamber. ON, olfactory nerve fascicles; NCM, naris constrictor muscle; PG, palatine ganglion; TN, nervus terminalis; VNN, vomeronasal nerve; VNO, vomeronasal organ. Bar = 500 μ m.

rons within the palatine ganglion of the salamander (Wirsig-Wiechmann and Ebadifar, 2002), which strongly suggests that these two neural populations belong to the nervus terminalis system.

In those species with distributed neurons, the neural cell bodies are frequently fusiform/bipolar cells (Fig. 5A) and are embedded within olfactory (Wirsig and Getchell, 1986), vomeronasal (Wirsig and Leonard, 1986) and autonomic nerve fascicles (Wirsig-Wiechmann and Lepri, 1991) in the nasal cavity. In the brain, the neural cell bodies are diffusely distributed along nervus terminalis fiber projections. Neurons in compact ganglia tend to be rounder and frequently larger (Oka, 1992; White and Meredith, 1995; Fig. 5B).

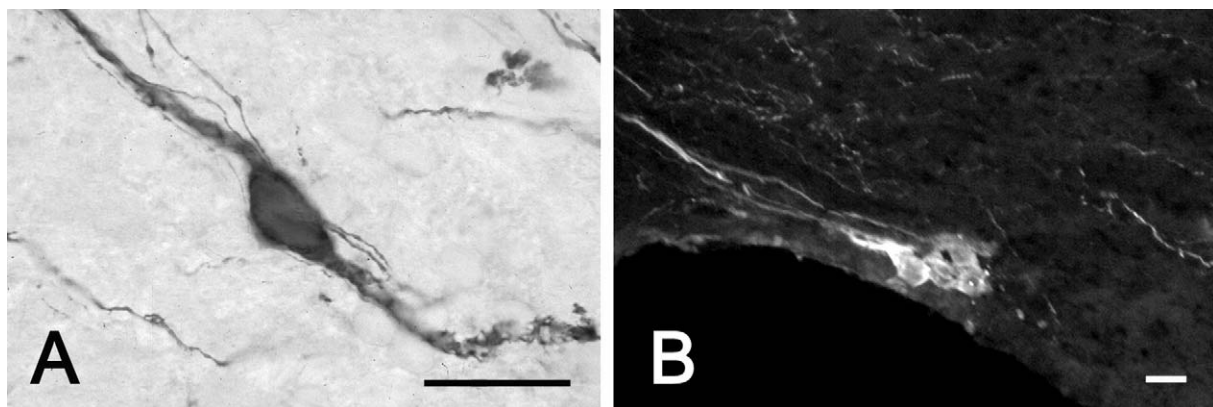


Fig. 5. Micrographs of GnRH nerve terminalis neurons from (A) tiger salamander, and (B) dwarf gourami illustrating the morphological differences of these neurons between the two species. (A) Salamander GnRH neuron from the ventral forebrain. Salamander GnRH neurons are usually medium-sized (roughly 20 μm) fusiform-shaped bipolar neurons with thick invaginated dendritic processes tapering from the cell body. (B) Gourami GnRH neurons in the nervus terminalis ganglion located at the ventrocaudal border of the olfactory bulb. The largest of the gourami nervus terminalis ganglionic neurons are round and 30 μm in diameter. Bars = 30 μm .

Nervus terminalis projections

Nervus terminalis neurons generally have two axons, one that projects peripherally toward the chemosensory mucosa, and one that projects toward the brain. The peripherally directed nervus terminalis axons project to a number of nasal structures including olfactory and vomeronasal mucosa, Bowman's glands, smooth naris muscle and autonomic parasympathetic ganglia (Wirsig and Getchell, 1986; Wirsig-Wiechmann and Lepri, 1991; Wirsig-Wiechmann, 1993; Wirsig-Wiechmann and Holliday, 2002). These processes do not appear to contact specific cells; rather they end within the lamina propria of chemosensory mucosa. A few processes appear to lie in proximity to Bowman's glands in the very rostral regions of the olfactory mucosa in salamanders (Wirsig-Wiechmann, 1993). Like the GnRH processes in the median eminence, the nervus terminalis fibers are frequently varicose, suggesting that GnRH is secreted along the length of the process. Ultrastructural evidence also suggests that GnRH may be secreted from the neural cell body of nervus terminalis neurons (Oka and Ickikawa, 1991). These data suggest that GnRH is acting as a paracrine hormone/modulator in the nasal cavity, autonomic ganglion and perhaps the brain.

Evidence that GnRH is secreted from the nervus terminalis in the nasal cavity is based on the ob-

servation that (1) olfactory and vomeronasal neurons possess GnRH receptors (Wirsig-Wiechmann and Jennes, 1993; Wirsig-Wiechmann and Wiechmann, 2001), and (2) GnRH modulates the manner in which chemosensory neurons respond to odors (Eisthen et al., 2000; Wirsig-Wiechmann et al., 2000). Gonadotropin-releasing hormone may reach the chemosensory neurons via diffusion directly from the nervus terminalis fibers locally, through intravascular transport and diffusion, or uptake and secretion by nasal glands (Fig. 6). The fact that chemosensory neurons do not show a response to GnRH if their cilia are removed (Rona Delay, personal communication) strongly suggests that GnRH must reach the surface of the mucosa. Nervus terminalis fibers do not project to the surface of the epithelium (which is sealed by tight junctions between chemosensory neuron dendrites) but are restricted to the lamina propria. We hypothesize that glandular secretion is the mechanism of GnRH transport to the cilia (Fig. 6).

Within the brain, nervus terminalis fibers generally project diffusely to widespread areas. In gourami fish, Oka and his colleagues (Oka, 1992; Oka and Matsushima, 1993) have shown via intracellular tracer injection that a single nervus terminalis neuron (located near the olfactory bulb) has a massive projection to multiple areas of brain and even into the brainstem. In some species of fish, neurons of

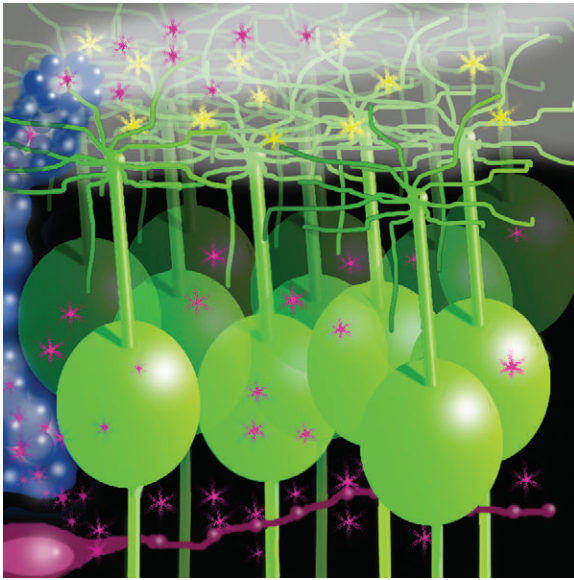


Fig. 6. Illustration of the chemosensory mucosa depicting two mechanisms by which GnRH could reach the chemosensory neurons (green neurons): (1) Diffusion of GnRH (pink stars) into the chemosensory epithelium from the GnRH neuron (pink neuron) located in the lamina propria (lowest black region). In this case GnRH would interact with receptors on the neural cell bodies or proximal dendrites. (2) Uptake of GnRH by nasal glands (blue gland) in the lamina propria and release from gland ducts along with glandular mucus (white mucus) onto the surface of the chemosensory epithelium (upper white region). In this case, GnRH would stimulate receptors on the dendritic knob or cilia/microvilli. On the surface of the chemosensory epithelium, which is adjacent to the lumen of the nasal cavity, the odor molecules (yellow stars) also interact with the olfactory receptor neuron cilia (depicted here) or vomeronasal neuron microvilli to stimulate odor receptors.

the nervus terminalis ganglion project through the brain, into the optic nerve and to the retina (Stell et al., 1984; Grober et al., 1987). In many studies the nervus terminalis neurons that project to the retina have been termed the *nucleus olfactoretinalis*. There is some controversy as to whether this group of neurons belongs to the nervus terminalis system (Szabo et al., 1991). In catfish, nervus terminalis neurons have been reported to project to the pituitary gland (Krishna et al., 1992). Several studies in amphibians have utilized tract tracers such as horseradish peroxidase to trace nervus terminalis projections to the brain (Hofmann and Meyer, 1989). However, most of the results remain inconclusive since extrabulbar

olfactory projections (Hofmann and Meyer, 1992) have also been labeled. Hofmann and Meyer (1992) have demonstrated labeled nervus terminalis neurons in the nasal cavity following injections of tracer into the diencephalon.

In mammals, very few definitive tracing studies have been carried out to determine how extensively an individual neuron projects. Jennes (1987) has conducted the only study that has shown the projections of an individual nervus terminalis neuron to the amygdala. Additionally, from numerous immunocytochemical studies, it is fairly clear that the nervus terminalis projects diffusely to olfactory bulb, rostral olfactory regions of the forebrain and septum (Schwanzel-Fukuda and Silverman, 1980; Witkin and Silverman, 1983; Jennes, 1986; Wirsig-Wiechmann and Wiechmann, 2001). More caudally it is not possible to differentiate nervus terminalis from preoptic GnRH neuron projections.

Neural interactions

Anatomical and electrophysiological data from fish and amphibians suggest that nervus terminalis neurons interact with one another (e.g. GnRH neurons can stimulate other GnRH neurons' activity; Abe and Oka, 2000) as well as their target organs (e.g. olfactory epithelium; Eisthen et al., 2000). In amphibians, we have observed a close relationship between GnRH neurons and NPY-like neurons (Fig. 7). This has also been observed in the nervus terminalis of a mammal, the brown bat (Oelschlager et al., 1998). Whether the NPY-like peptide influences GnRH neural function and vice versa in the nervus terminalis is not known. However, there is evidence that NPY and GnRH neurons of the preoptic/hypothalamic system interact with one another as well as on their target, the pituitary gland. Neuropeptide Y increases GnRH secretion in the preoptic/hypothalamic system (Crowley and Kalra, 1987). It also influences luteinizing hormone secretion by itself, and pre-exposure to NPY has been shown to augment pituitary cell responses to GnRH (Evans et al., 2001). Interestingly, in catfish, the NPY-like fibers project to the pituitary (Krishna et al., 1992) and may interact with the GnRH fibers in control of gonadotropin release. In relation to the innervation of sensory targets by the nervus terminalis system,

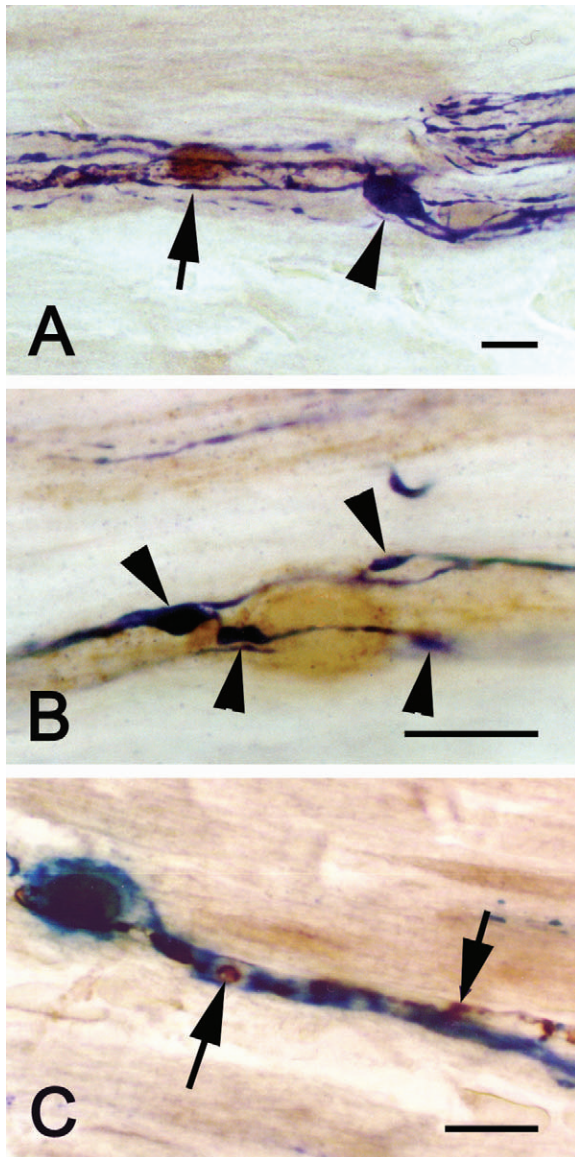


Fig. 7. Micrographs of tiger salamander olfactory nerve fascicles including nervus terminalis neurons labeled with GnRH (brown) and FMRFamide (blue) antisera. (A) Two nervus terminalis neurons within the plexus, one containing GnRH (arrow) and the other an NPY/FMRFamide-like peptide (arrowhead). (B) Apparent contacts (arrowheads) by an NPY/FMRFamide-like neuron onto a GnRH neuron. (C) Apparent contacts (arrows) by a GnRH neuron onto an NPY/FMRFamide-like neuron. Axonal contacts between neurons generally occur on the cell body (B) or dendrite (C). Bars = 20 μ m.

GnRH and the NPY-like peptide individually can modify the activity of peripheral sensory systems (olfactory receptor neurons: Eisthen et al., 2000, 2001; and retinal ganglion cells: Stell et al., 1987).

In some fish (e.g. goldfish, dwarf gourami) the GnRH and NPY-like peptide are in the same nervus terminalis neurons and therefore could be released together (Wirsig-Wiechmann and Oka, 2002). There may be some intra-neuronal control over the relative amount of each peptide released by factors such as the firing frequency of the neuron. The separation of GnRH and NPY-like neural populations in other species undoubtedly enables greater neural and/or hormonal control of release of the two peptides, giving the system greater flexibility in its response to inputs from different sources (White and Meredith, 1995).

Nervus terminalis neurons receive inputs from multiple brain areas (Fernald and Finger, 1984; Yamamoto and Ito, 2000). The brain areas that project to the nervus terminalis ganglion mainly include sensory processing regions, especially for olfactory information, and projections from brainstem modulatory systems. It has been shown that the nervus terminalis ganglion in sharks specifically receives noradrenergic inputs from central sources (Fernald and Finger, 1984) and that these inputs influence the activity of the nervus terminalis ganglion neurons (White and Meredith, 1995). Axonal inputs to the nervus terminalis mainly contact the proximal portion of the dendrite and the cell body (Figs. 7 and 8). These inputs have been shown to involve synaptic contacts with nervus terminalis neurons (White and Meredith, 1987; Oka and Ickikawa, 1991). While it is known that adjacent GnRH nervus terminalis neurons can influence the activity of one another (Abe and Oka, 2000; Oka and Abe, 2001), very little is known about the specific stimuli that control the activity level of the nervus terminalis. Nervus terminalis GnRH neurons do not appear to be activated at the time of the luteinizing hormone surge and ovulation (Lee et al., 1990). However, the synthesis and/or release of GnRH in these neurons are influenced by steroids (Wirsig-Wiechmann and Lee, 1999).

Based on neuroanatomical data collected on the location of nervus terminalis cell bodies, axonal projections and interactions with other neural systems, we propose the following minimal characteristics in

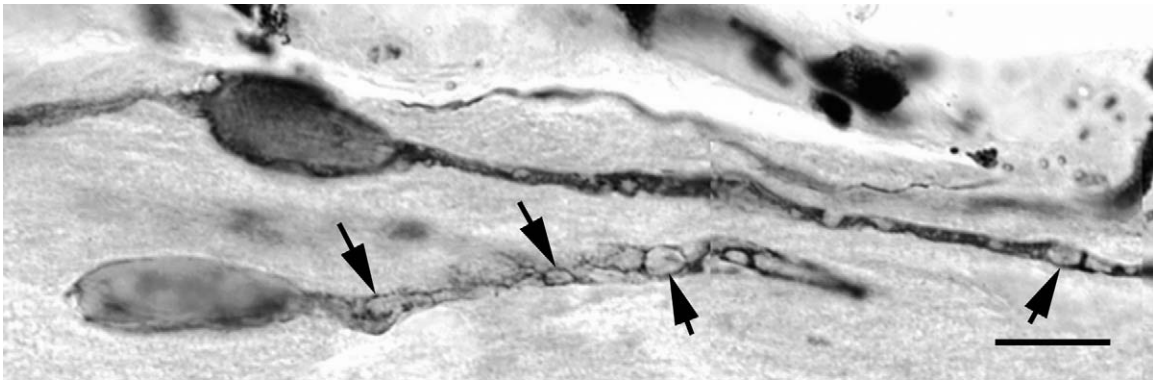


Fig. 8. Micrographs of two tiger salamander nervus terminalis neurons located within the olfactory nerve illustrating invaginations (arrows) within the dendrites. These invaginated areas appear more lightly labeled because there is little cytoplasm in this area. At these invagination sites presumed axonal contacts occur. The upper neuron contains GnRH and the lower neuron contains an NPY/FMRamide-like peptide. Bar = 20 μ m.

defining the nervus terminalis: (1) its cell bodies lie either within the nasal cavity or within the region of the olfactory bulbs, (2) its axons project peripherally to the chemosensory and/or nasal mucosa and centrally to ventral forebrain areas, especially olfactory/limbic/hypothalamic areas, and (3) its cell bodies receive neural inputs from central brain regions.

Species diversity

The functional studies of the nervus terminalis system all suggest that it is a neuromodulatory system involved in reproduction. While certain features of the nervus terminalis are consistent across all species (e.g. presence of GnRH), there are some obvious species differences in the location, density, morphology and size of nervus terminalis cell bodies, relative proportion of GnRH containing neurons compared to non-GnRH containing neurons, and axonal projections. These differences may not reflect significant functional differences of the system, but rather differences in species' body forms and specific behavioral/endocrine strategies related to reproduction.

There is variation in the proportion of nervus terminalis neurons that contain GnRH as opposed to other neurotransmitters among different species. While in certain species of fish (e.g. dwarf gourami), the GnRH to non-GnRH ratio of terminal nerve neuron number is roughly 1:2 (Oka et al., 1986), in rodents the ratio is almost five times as great

(Schwanzel-Fukuda and Silverman, 1980; Wirsig and Leonard, 1986). Our studies in hamsters suggest that approximately 10% of nervus terminalis neurons contain GnRH (see Fig. 3). However, in tiger salamanders, it appears that equal numbers of neurons contain GnRH or a FMRamide/NPY-like peptide (unpublished observations; Fig. 7).

The size of the neurons along the nervus terminalis also varies between species. In dwarf gourami the neurons of the terminal nerve ganglion can be as large as 30 μ m (Oka and Ickikawa, 1991; Fig. 5), whereas in many other species the neuronal diameter averages from 10 μ m in rodents (Fig. 3) to 20 μ m in amphibians (Fig. 7). The cell size may reflect the extensiveness of the projections of individual nervus terminalis neurons. In fish, a single neurons can send projections to many parts of the brain, including the brainstem (Oka, 1992). In mammals, it appears that the nervus terminalis projects to more rostral brain areas including the olfactory, limbic and hypothalamic regions rather than brain stem areas (Jennes, 1987). In many species, the non-GnRH containing neurons generally tend to be larger (1.5 \times) than the GnRH containing neurons (Schwanzel-Fukuda and Silverman, 1980), suggesting a difference in function or projections compared to the GnRH containing neurons.

The nasal regions to which the nervus terminalis projects differ somewhat between species. In rodents, the nervus terminalis is associated with the vomeronasal system (Fig. 1). The GnRH contain-

ing fibers of the nervus terminalis project mainly to the lamina propria of the vomeronasal organ. In addition, GnRH fibers from neurons located in the pterygopalatine ganglion project along the nasopalatine nerve to innervate the caudal region of the vomeronasal organ. This association of the nervus terminalis with the vomeronasal organ correlates with the fact that this chemosensory organ is very important in rodent reproduction (Wysocki, 1979). In tiger salamanders, while some GnRH fibers project to the vomeronasal organ, most nervus terminalis processes are confined to ventromedial olfactory nerve bundles that project to the rostromedial region of the olfactory chamber (Fig. 4). It is unclear whether this region of the chemosensory mucosa is involved in reproduction, such as the detection of pheromones.

It is interesting to note that, in tiger salamanders, the rostral olfactory epithelium is thicker, and demonstrates a much slower cell turnover rate and is less responsive to odors than the caudal olfactory epithelium (MacKay-Sim and Patel, 1984). A positive correlation between GnRH administration and inhibition of cell growth has been demonstrated in cancer therapies (Ben-Yehudah et al., 2001; Grundker et al., 2001). It is possible that an increase in GnRH secretion from the nervus terminalis during mating could influence cell turnover in the chemosensory epithelium in addition to its immediate effect on olfactory receptor neuron responses to odors (Eis-then et al., 2000). It could be argued that high cell turnover might not be advantageous during courtship and mating when an animal needs to conserve energy and use the sensory system extensively for pheromonal detection. Dawley and coworkers (Dawley, 1998; Dawley et al., 2000) have shown that there are seasonal changes in chemosensory epithelium thickness and vomeronasal organ size in the salamander, *Plethodon cinereus*. Cell turnover is highest at the end of the mating season. However, it is not clear whether these changes are related directly to GnRH secretion from the nervus terminalis.

In certain species of fish, the nervus terminalis sends projections through the brain to the retina. These projections include both the GnRH and FMRFamide/NPY-like components of the nervus terminalis system. While such direct projections to the retina do not appear to be very common in

other classes of animals, there have been reports of GnRH fibers in the optic nerves of mammals (Witkin, 1987, in primates; Wirsig-Wiechmann and Wiechmann, 2002, in voles). These GnRH fibers do not seem to project directly into the retina. The retina of voles does express GnRH receptors (Wirsig-Wiechmann and Wiechmann, 2002) suggesting that the GnRH reaches the retina by diffusion along the optic nerve or through the vasculature. In *Rana pipiens*, the FMRFamide/NPY-like (but not the GnRH) component of the nervus terminalis sends fibers to the retina (Wirsig-Wiechmann and Basinger, 1988).

In the majority of animals, the fibers of the nervus terminalis are unmyelinated, secretory fibers. However, in sharks and dolphins, many of the nervus terminalis fibers are myelinated (White and Meredith, 1987; Demski et al., 1987). It has been proposed that the non-GnRH population of nervus terminalis neurons may be autonomic (Buhl and Oelschlager, 1986; Meredith and White, 1987), which is supported by the fact that these neurons appear to be cholinergic (Schwanzel-Fukuda et al., 1986; Wirsig and Leonard, 1986).

It is interesting to note that while whales and dolphins completely lack a nasal chemosensory system, they still possess a nervus terminalis. With respect to these animals, the potential autonomic component of the nervus terminalis may have become adapted for diving, and/or controlling nasal gland secretion or vascular tone rather than for modulating olfaction (Buhl and Oelschlager, 1986). The intracerebral GnRH component may still be functional in cetaceans since it is thought to modulate brain areas subserving reproductive functions.

Conclusion

The nervus terminalis is a neurosecretory modulatory system that receives input from the brain and modulates peripheral sensory systems and brain nuclei probably related to reproduction. This system does exist in the human nasal cavity. It may participate in apparent alterations in olfactory sensitivity related to changes in circulating hormones (Doty et al., 1981; Hummel et al., 1991; Graham et al., 2000) as well as gender-specific activation of reproductive brain areas by pheromonal stimulation (Savic et al., 2001; Sobel and Brown, 2001). Understanding the

manner in which the nervus terminalis might influence our responses to social odors may shed light on the subtle aspects of human behaviors such as the choice of a partner.

Based on the research conducted to date on this system in animals as well as in humans, we propose the following defining characteristics for the nervus terminalis neurons: The neurons: (1) arise from the neural crest and migrate from the olfactory placode region to nasal and rostral brain areas, (2) in the mature organism, lie within the nasal cavity or superficially in the substance of the brain near the olfactory bulbs and receive inputs from the brain, (3) project to nasal mucosa and rostroventral areas of the brain, especially olfactory/limbic areas, and (4) contain GnRH, acetylcholine and an NPY-like peptide. It is clear from the studies conducted over the past 20 years, that the nervus terminalis projects to and modulates a number of peripheral and central structures involved with sensory processing and reproduction. The peripheral systems that receive the greatest and most direct projections from the nervus terminalis appear to be the most important for the reproductive strategies of the species (e.g. in fish that use visual information for mating the nervus terminalis projects extensively to the retina; in rodents that use pheromonal information for mating the nervus terminalis projects extensively to the vomeronasal organ). The purpose of this initial attempt to define the characteristics of the nervus terminalis as a functional unit is to set a basic framework for designing experiments that can test hypotheses regarding the mechanisms by which the nervus terminalis influences and is influenced by other neural systems.

Abbreviations

GnRH	gonadotropin-releasing hormone
FMRFamide	molluscan cardioexcitatory peptide
NPY	neuropeptide Y

Acknowledgements

The research cited here by Wirsig or Wirsig-Wiechmann was supported largely by grants from NIH (NS27586 and DC04270), NSF (IBN-9896098), Presbyterian Health Foundation and the Oklahoma Center for the Advancement of Science

and Technology (HR00-078). Heather L. Eisthen is supported by NSF grant IBN 9982934.

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CHAPTER 5

Angiogenesis in association with the migration of gonadotropic hormone-releasing hormone (GnRH) systems in embryonic mice, early human embryos and in a fetus with Kallmann's syndrome

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Introduction

The purpose of this review is to examine the theoretical notion that development of the vasculature in the head, and in particular, in the nasal mesenchyme, may play a role in the migration and ultimately, in the function of the gonadotropin-releasing hormone (GnRH), or luteinizing hormone-releasing hormone (LHRH) systems in mammals. From early embryogenesis, fetal development and birth, through puberty and generation of the reproductive cycles in both sexes, a close association is consistently found between the GnRH cells and the blood vessels (Silverman, 1988). The GnRH neurosecretory cells, in response to blood borne secretions of estrogen by the ovaries or of testosterone by the testes, release their hormone into the vasculature of the primary portal plexus of the median eminence. Circulating through the sinusoids of the anterior pituitary gland, the GnRH is taken up by receptors on the gonadotrops which evoke the secretion of luteinizing hormone (LH) or of follicle-stimulating hormone (FSH) into

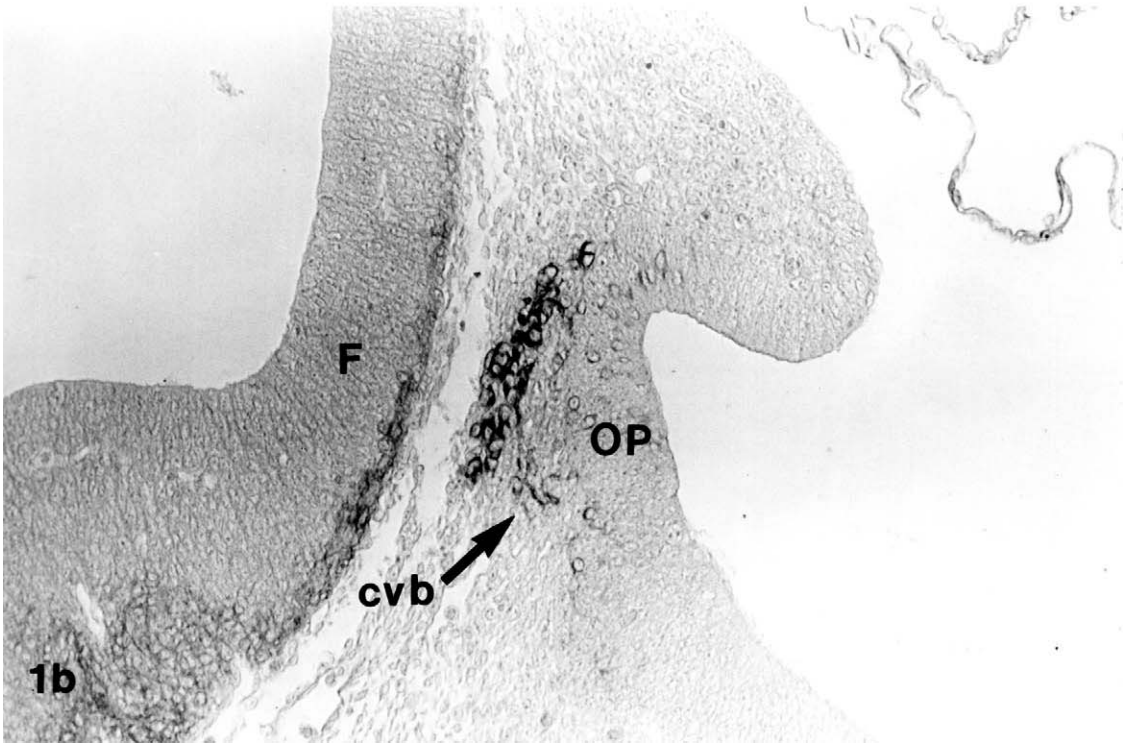
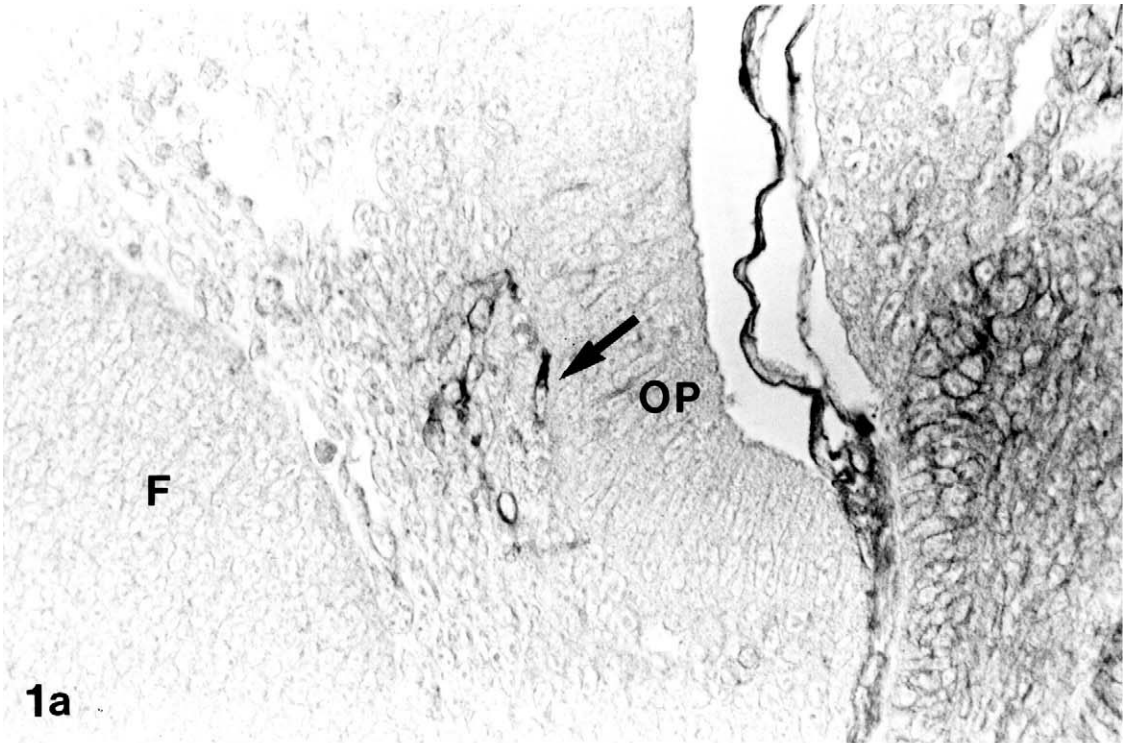
the bloodstream. Every cell along this vascular route is exposed to these hormones, but only those cells in the ovaries and the testes, which contain the required receptors, are activated. In response, steroid hormones are released into the blood. In turn, the steroid hormones are bound by receptors in neuroendocrine cells and directly by receptors and in the gonadotrops of the anterior pituitary gland. The vasculature is thus an integral and essential part of the GnRH systems, from the nascent formation of the migration route and the origin of GnRH cells in the olfactory placode through the actual mediation of reproductive functions (Pfaff and Schwanzel-Fukuda, 1995).

Observations in mice

(a) Formation of the cellulovascular bridge and aggregate below the rostral tip of the forebrain visualized by N-CAM immunoreactive axons and cell bodies from the epithelium of the olfactory placode. (b) The initial appearance of GnRH-immunoreactive neurons in the epithelium of the olfactory pit. (c) Organization of the GnRH cell migration route along the N-CAM-immunoreactive axons in contact with the rostral forebrain is concomitant with the formation of the olfactory bulb.

In embryonic mice, on days 9 through 10 of gestation (22 to 26 pairs of somites are present) the ol-

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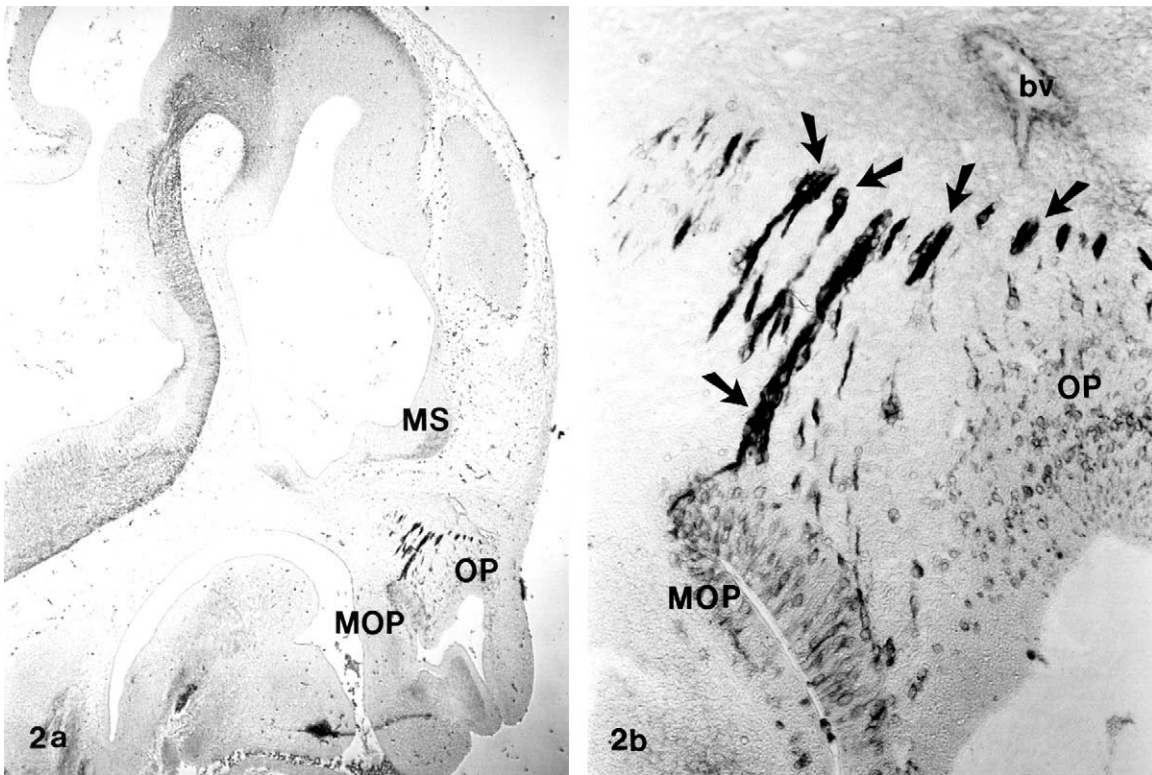


Fig. 2. (a) Photomicrograph of an 8 μ m sagittal section through the head of a 12-day-old embryonic mouse at low (a) and higher (b) magnification. This section is just on midline, between the forebrain vesicles and includes a part of the developing medial septal region of the brain (MS), the olfactory pit (OP) and the medial olfactory pit (MOP), the anlage of the vomeronasal organ. (b) The cellulovascular bridge is seen here in its most medial aspect, and N-CAM-immunoreactive axons and cell bodies form a part of the migration route for GnRH neurons (arrow).

factory placodes (thickenings of the ectoderm on the ventrolateral sides of the developing head) invaginate to form simple olfactory pits either side of midline (Fig. 1a, Schwanzel-Fukuda et al., 1992). Soon after, a secondary recess, the anlage of the vomeronasal organ, forms in the medial wall of either olfactory pit. Within the luminal, intermediate and basal layers of the olfactory epithelium N-CAM immunoreactive cells are visible. The N-CAM-immunoreactive ax-

ons, soon followed by N-CAM-immunoreactive cell bodies emerge from the basal layer of this epithelium into the nasal mesenchyme where they aggregate around the numerous small blood vessels (Fig. 1a, b) which are present in this part of the nasal mesenchyme between the olfactory pit and the developing forebrain (Schwanzel-Fukuda et al., 1992, 1996). These N-CAM-immunoreactive axons and cell bodies and the blood vessels make up the cellulovas-

Fig. 1. (a) Photomicrograph of an 8 μ m sagittal section through the head of an early 10-day-old embryonic mouse. The olfactory placode has invaginated to form a simple olfactory pit (OP). A fusiform N-CAM-immunoreactive cell (arrow) is seen at the basal surface of the epithelium and in this plane of section five lightly stained N-CAM-immunoreactive cells are seen nearby. At this age the forebrain (F) is comparatively close to the developing olfactory pit. (b) Photomicrograph of an 8 μ m section through the head of a 10-day-old embryonic mouse, a little older than that seen in Fig. 1a. The olfactory pit (OP) is more deeply invaginated and several lightly stained N-CAM-immunoreactive cells are seen in the intermediate and basal strata of its epithelium. Darkly stained N-CAM-immunoreactive cells form a cellular aggregate in the nasal mesenchyme. A few N-CAM-immunoreactive fibers (arrow) extend from the epithelium of the olfactory pit into the cellular aggregate, the beginning of the formation of the cellulovascular bridge (CVB).

cular bridge, 'strand' (Bossy, 1980) or 'blastema' (Lejour, 1967) seen in the nasal mesenchyme, coursing from the epithelium of the medial olfactory pit, across the developing nasal septum, into the forebrain (Fig. 2a, b). This cellulovascular bridge (CVB), the medial part of which ultimately forms the migration route for the GnRH neurons, is made up (from medial to lateral) of N-CAM-immunoreactive axons of the nervus terminalis, the vomeronasal and the olfactory nerves (Schwanzel-Fukuda and Pfaff, 1989; Schwanzel-Fukuda et al., 1992). The N-CAM-immunoreactive cell bodies form the cellulovascular aggregate, actually a part of the cellulovascular bridge, seen just below the anlage of the forebrain (Fig. 3). This aggregate of olfactory placode-derived cells includes the N-CAM immunoreactive axons of the olfactory, vomeronasal and terminalis nerves, and the cell bodies of the Schwann cells which accompany them throughout their peripheral distribution. A population of N-CAM immunoreactive ganglion cell bodies associated with the terminalis nerve are seen in the *ganglion terminale* (Huber and Guild, 1913) the largest ganglion of this nerve. It is seen on the most medial side of the cellulovascular aggregate, either side of midline, usually above the anlage of the cribriform plate at later stages (from days 14) of gestation. Smaller ganglia are seen at nodal points along the course of the nervus terminalis across the nasal septum, through the cribriform plate and by three or four short central roots into the medial forebrain and preoptic areas.

GnRH-immunoreactive neurons, in mice, are first detected by immunocytochemical procedures and antibodies to GnRH (Fig. 4) at about 11 to 11.5 days of embryogenesis (more than 45 pairs of somites are present at this age). The GnRH-immunoreactive neurons are found in the epithelium of the medial part of the olfactory pit, either side of midline (Schwanzel-Fukuda and Pfaff, 1989; Wray et al., 1989). Widely separated from each other throughout the placodal epithelium, as the GnRH neurons emerge into the nasal mesenchyme they form cords along, or surrounded by, N-CAM-immunoreactive axons of the terminalis and vomeronasal nerves and perforating branches of the nasal arteries and veins (Fig. 2a, b). The GnRH cells are never found independent of these axons in the nasal mesenchyme. Thus, they begin their migration across the nasal

septum. In transverse section they can be seen on the most medial parts of the cellular aggregates either side of midline (Fig. 3) and can be followed into the medial sides of the cerebral hemispheres where they enter the brain. In sagittal section the GnRH-immunoreactive cells enter the ventromedial forebrain, caudal to the anlage of the olfactory bulb, and course along the medial surfaces of the cerebral hemispheres to enter the septal and preoptic areas of the brain (Fig. 5a and b). While on the nasal septum, the majority of the nervus terminalis ganglion cells, including those of the large ganglion terminale are N-CAM immunoreactive and appear to outnumber the GnRH cells. By day 12 of embryogenesis in mice (48–52 pairs of somites are present), olfactory axons have made contact with the rostral forebrain and the anlage of the olfactory bulb can be recognized. Soon after, from about day 13.5 (60 pairs of somites present at this age) through day 16, the part of the N-CAM-immunoreactive cellulovascular aggregate that receives the olfactory nerves *en route* to the olfactory bulb develops into the olfactory nerve layer of the olfactory bulb (Schwanzel-Fukuda et al., 1992). Schwann cells are seen among the axons of the olfactory nerves in this layer. The distinctive cellular morphology of the olfactory bulb soon develops, and the accessory olfactory bulb receives the incoming axons of the vomeronasal nerves and a recurrent branch of the nervus terminalis. Three or four central roots of the nervus terminalis, the most medially placed of the olfactory placode-derived axons, enter the septal and preoptic areas of the ventromedial forebrain, caudal to the olfactory bulbs (Fig. 5a, b). In mice, the migration of GnRH cells into the brain is largely over by 16 days of gestation (Schwanzel-Fukuda and Pfaff, 1989).

Formation of the meninges in mice and development of the vasculature

(a) The leptomeningeal (pia and arachnoid matres) plexus of blood vessels along the ventromedial surface of the brain. (b) The pachymeninx (dura mater) and formation of the dural venous sinuses. (c) The initial appearance of nucleated red blood cells.

In 10-day-old embryonic mice (30–34 somites are present at this age), the anterior and posterior neuropores have closed and the neural tube is now



Fig. 3. Photomicrograph of an 8 μ m coronal section through the head of a 13-day-old embryonic mouse. At this age the nuclei of the N-CAM immunoreactive cells are seen in the middle or intermediate zone of the epithelia of the olfactory pits, in contrast the location of these nuclei in the 11- to 12-day-old embryonic mouse. The N-CAM-immunoreactive axons and cell bodies are coursing from the lateral and dorsal parts of the placodal epithelium into the N-CAM-immunoreactive cellular aggregates seen just below the anlagen of the forebrain (F). GnRH-immunoreactive cells migrate toward the brain along the medial part of the cellulovascular bridge and aggregate (arrows) and some cell have reached the medial surfaces of the cerebral hemispheres (arrows).

completely closed (Kaufman, 1998). The brain then undergoes a rapid development which is paralleled by the development of the cranial blood vessels, which soon “invest the central nervous system with

an extensive vascular plexus” (Theiler, 1989). As early as 8.5 days of gestation in mice (8 somites are present) a vascular anastomosis is present between the two dorsal aortae and the unpaired omphalo-

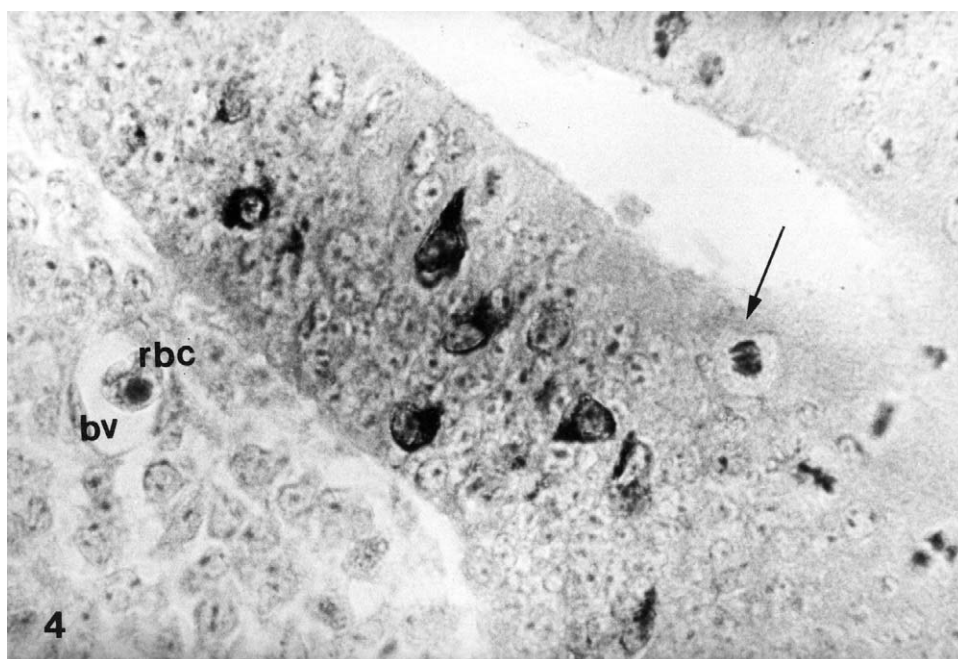


Fig. 4. Photomicrograph of an 8 μ m sagittal section, at high magnification, showing the initial detection of GnRH-immunoreactive cells (black) in the epithelium of the medial olfactory pit of an 11.5-day-old embryonic mouse. This section has been lightly stained with cresyl violet. Near the luminal surface, a cell is seen in mitosis (arrow). Small blood vessels (bv) are seen along the basal surface, one of which contains a nucleated red blood cell (rbc).

mesenteric trunk which formed from fusion of the paired vitelline arteries. The embryonic and yolk sac circulations are now in direct communication and a small number of nucleated red blood cells are seen in the embryonic vasculature (Kaufman, 1998). Remarkably, this vascular anastomosis will go on to form the unpaired abdominal aorta, and the singular celiac and superior and inferior mesenteric arteries which branch extensively from it (Theiler, 1989; Kaufman, 1998) to supply blood to the abdominal viscera of the digestive system.

The blood vessels which proliferate in the nasal mesenchyme in mice, from days 9.5 (15 to 20 pairs of somites) on, appear to result from an outgrowth of this investing vascular plexus including branches of the internal carotid arteries which developed from the first and third aortic arch arteries (Fig. 6a and b and see Sadler, 1985). *Thus, angiogenesis rather than de novo synthesis or vasculogenesis in the nasal mesenchyme probably accounts for the proliferation of small vessels in precisely that area of the nasal mesenchyme which receives the emerging N-CAM*

immunoreactive axons and cell bodies and gives origin to (1) the cellulovascular bridge (CVB) and (2) the cellular aggregate. We hypothesize that these structures are crucial for dispatching the olfactory, the vomeronasal and the terminalis nerves (and the GnRH neurons) to their appropriate destinations in the brain. Lejour (Lejour, 1967) in a study of the development of the olfactory nerves in the rat, using alkaline phosphatase histochemistry, opines "... it is a mass of neuroblast-like cells to which all the neural fibres originating in the nasal epithelium and the Jacobson organ converge ... this blastema thus appears as a large ganglionic relay in the course of the olfactory fibres."

The loose mesenchyme which surrounds the developing brain and spinal cord, a forerunner of the leptomeninges (the pia and arachnoid maters) is believed by most developmental anatomists to be derived from the neural crest (O'Rahilly and Muller, 1986). The blood vessels which lie directly adjacent to the brain are considered to be the first pial vessels (Figs. 1a, b and 6a, b). With further growth

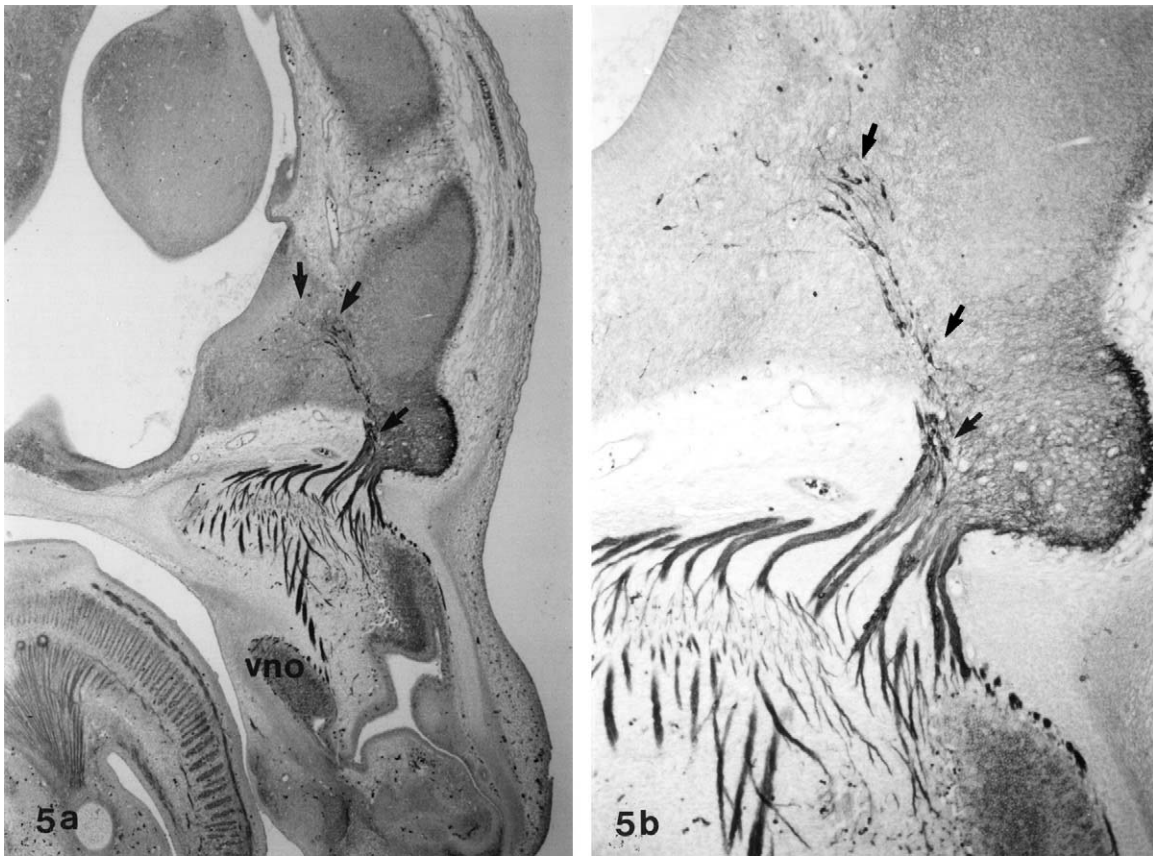


Fig. 5. (a) Photomicrograph of an 8 μ m sagittal section through the head of a 14-day-old embryonic mouse. N-CAM-immunoreactive cells are seen in the epithelium of the vomeronasal organ (vno), in cords on the nasal septum and entering the ventromedial forebrain with axons of the nervus terminalis, caudal to the olfactory bulbs. (b) On the nasal septum and in arc through the forebrain, GnRH-immunoreactive cells (arrows) accompany the N-CAM-immunoreactive axons. Please see the color plate, Fig. 16 (p. 76), for greater detail of this figure.

a membrane or cellular sheet, the pia mater, develops between the blood vessels and the cerebral wall. The leptomeningeal meshwork develops in the mesenchyme immediately adjacent to the brain, giving rise to a pia-vascular layer and an overlying fluid-filled subarachnoid space beneath the arachnoid layer of the meninges (Weed, 1917). This part of the cranial leptomeningeal meshwork is considered the “central part of the primitive or primary meninx” (O’Rahilly and Muller, 1986). The third layer of the meninges, the pachymeninx or dura mater, is not derived from the neural crest but from the mesoderm. This is the outer most layer of the meninges and it is present in the 14-day-old embryonic mouse (Kaufman, 1998). It lines the cranial vault and dou-

ble layers of the dura mater form the falx cerebri, the tentorium cerebelli and the dural sinuses including the superior, the inferior and the transverse venous sinuses (Theiler, 1989; Kaufman, 1998) which receive the venous blood from the brain and the cerebral spinal fluid from the subarachnoid spaces.

Observations of the development of the GnRH systems and meninges in human embryos. Comparisons with those seen in mice

In human embryos, the development of the N-CAM-immunoreactive migration route: the formation of the cellulovascular bridge (CVB), the cellular aggregate, and the origin and migration of the GnRH

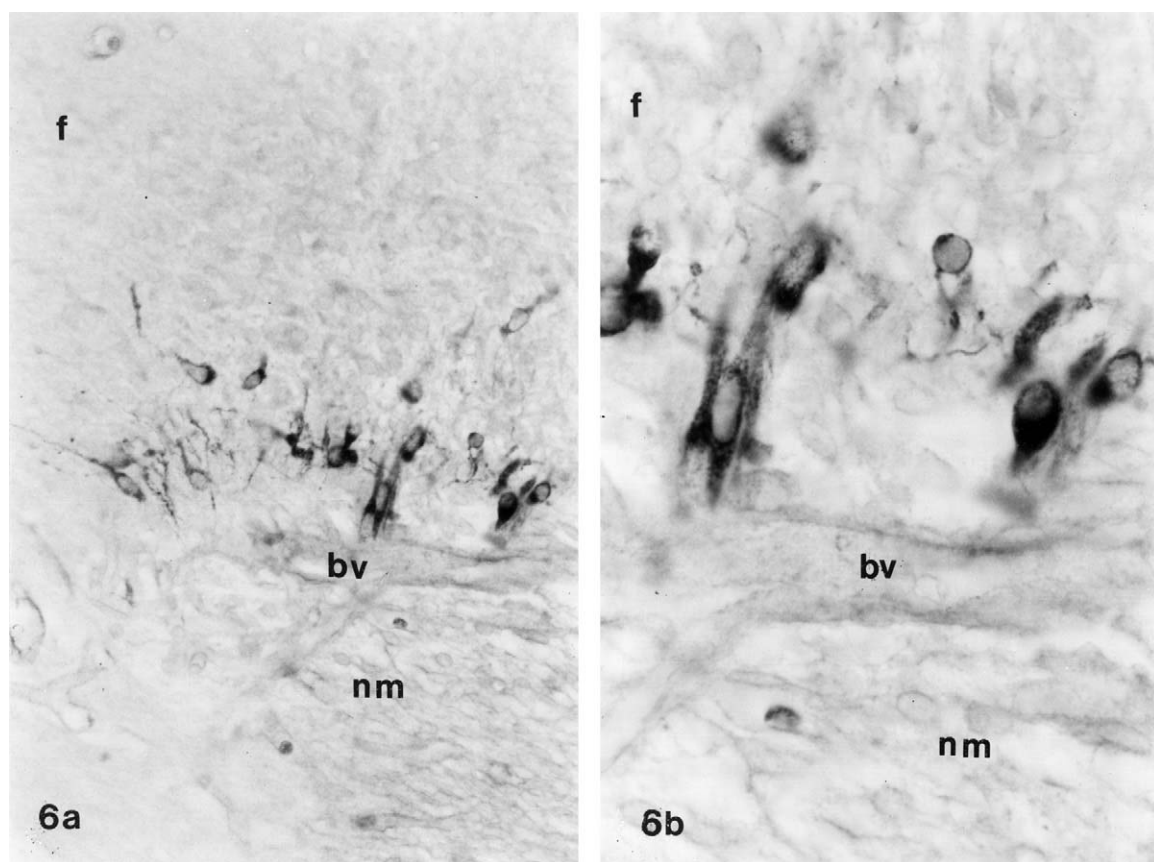
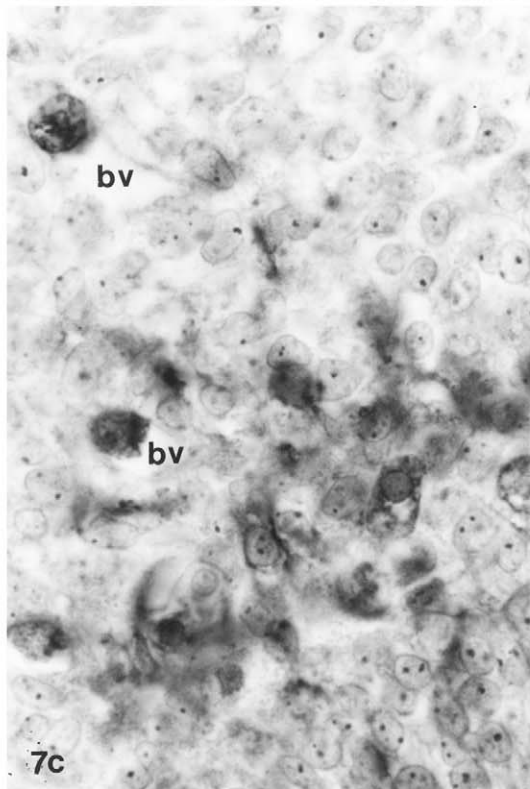
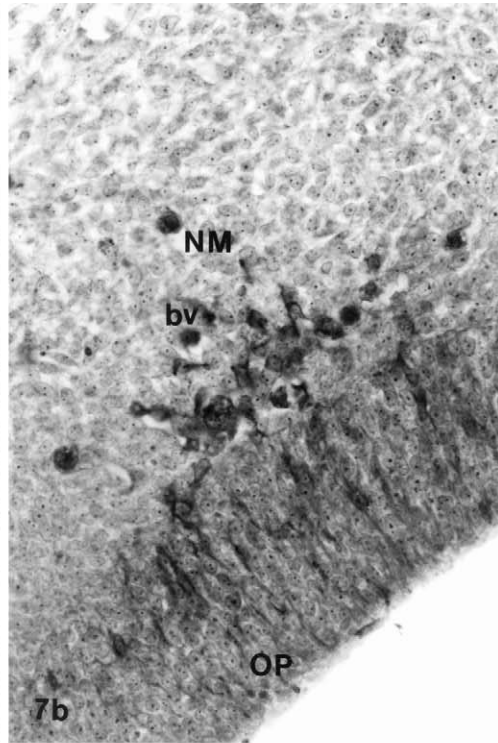
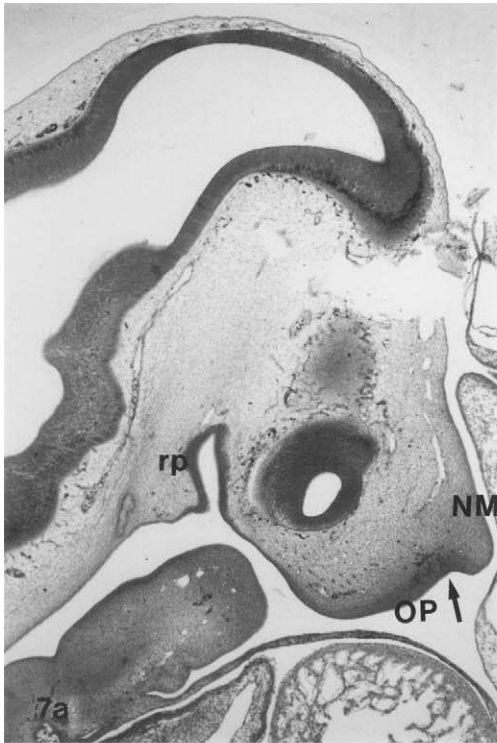


Fig. 6. (a) Photomicrograph of an 8 μ m sagittal section through the base of the ventromedial forebrain (f) of a 16-day-old embryonic mouse. At low magnification, a number of GnRH-immunoreactive cells (darkly stained with DAB chromogen, arrows) are seen emerging from the nasal mesenchyme in close association with small blood vessels (bv) containing vascular endothelial growth factor (VEGF)-labelled endothelial cells. (b) At higher magnification, these blood vessels appear to be opening into the parenchyma of the brain (arrows).

neurons into the brain is very similar to that seen in mice and other mammalian embryos. Note, that the GnRH-immunoreactive cells are seen by immunocytochemical procedures entering the ventromedial surface of the brain, caudal to the medial sides of the olfactory bulbs, either side of midline. They enter

the brain with the central processes of the terminal (and to some extent the vomeronasal) nerves. While there are species specific differences in the gestational ages of these events, the stages at which particular developments take place in mice and in human embryos are generally equivalent.

Fig. 7. (a) In an 8 μ m sagittal section through a whole 28-day-old human embryo, N-CAM-immunoreactivity is seen in all strata of the epithelium of the simple olfactory pit (OP) and a few N-CAM cells have emerged into the nasal mesenchyme (NM). At this age Rathke's pouch is present and open. Numerous small blood vessels are seen in the nasal mesenchyme between the olfactory pit and the developing forebrain (f). At higher magnification of this section (b and c) we trace the association of N-CAM-immunoreactive cells with the developing blood vessels (bv) in the nasal mesenchyme (NM) and the arrangement of the N-CAM cells in the epithelium of the olfactory pit. Note that these N-CAM immunoreactive cells are only present in the deepest part (arrow) of this olfactory pit. In (c) the nucleated red blood cells can be seen in the walls of the small blood vessels (bv) in the nasal mesenchyme. The N-CAM-immunoreactive cells consistently appear to aggregate around these vessels. No GnRH-immunoreactive cells are present in the 28-day-old human embryo.



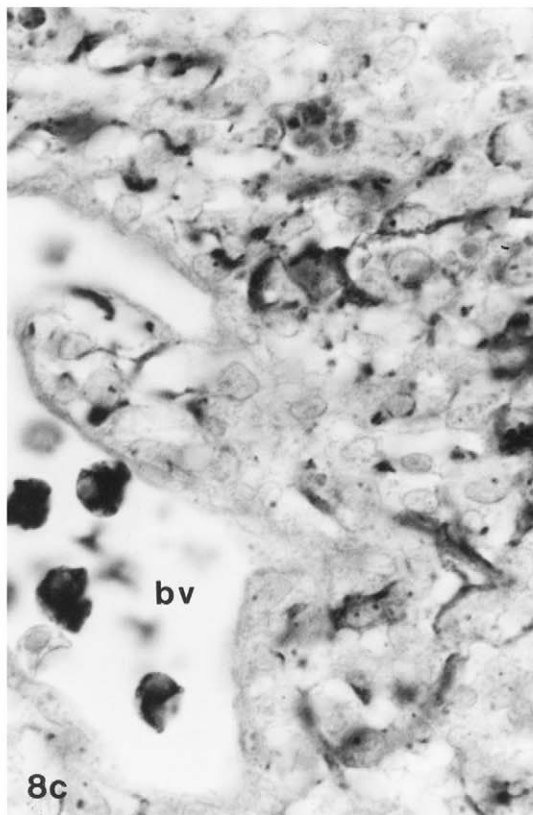
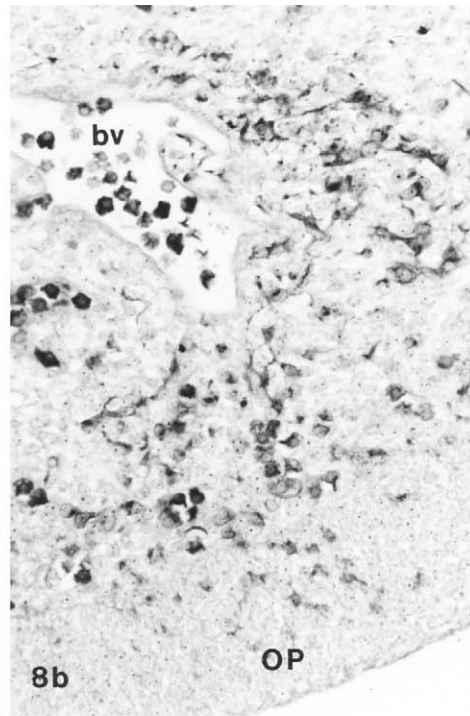
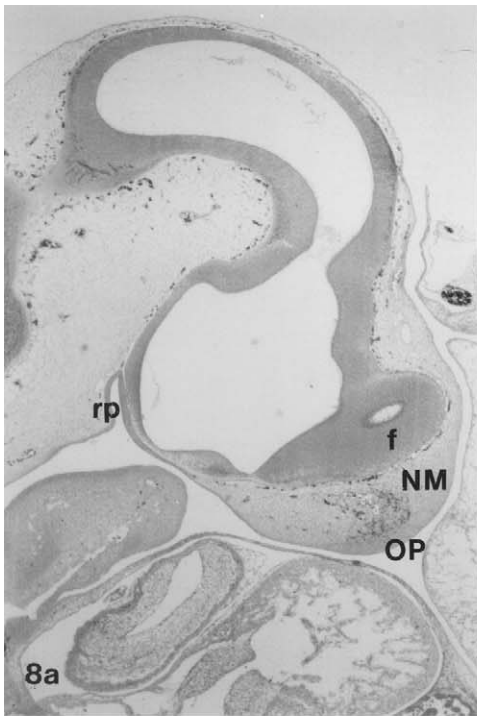
Briefly, in human embryos, at about 28 days of gestation (30 or more pairs of somites), both the anterior and the posterior neuropores are closed and the olfactory placode invaginates to form a simple olfactory pit (Fig. 7a–c; and see Pearson, 1941; Schwanzel-Fukuda et al., 1996). As the brain now begins to rapidly differentiate, a vascular meningeal plexus forms along the surface of the brain. At the same time, blood vessels elaborate in the nasal mesenchyme (Weed, 1917) from primitive branches of the first and third aortic arches. These vessels are the precursors of the sphenopalatine branch of the maxillary artery and the anterior and posterior ethmoidal branches of the ophthalmic artery. Branches of these arteries and their corresponding veins form dense arteriovenous anastomoses, actually “... a very fine network in the mucous membrane ... and a rich cavernous plexus well-marked over the inferior concha and lower part of the {nasal} septum.” (Lockhart et al., 1969). The arterial blood is thus shunted rapidly into the venous circulation of the head.

N-CAM-immunoreactivity is visible in the cells in the luminal, intermediate and basal parts of the epithelium of the medial part of the olfactory pit (Fig. 8a–c), a structure which will later form the vomeronasal organ. As in mice, N-CAM-immunoreactive axons, followed by the N-CAM-immunoreactive cell bodies from the epithelium of the olfactory pit, migrate into the nasal mesenchyme (Fig. 9a, b). Clustering around the nasal blood vessels, these axons and cell bodies form a distinctive cellulovascular bridge below the anlage of the rostral forebrain, the medial part of which will eventually serve as the migration route for GnRH cells into the brain in older embryos. The structure of the cellulovascular bridge in older human embryos 32, 42, and 46 days, as studied by light microscopy in this lab (Schwanzel-Fukuda et al., 1996), is larger in proportion to the nasal mesenchyme and appears to be

more compact than that seen in mice (compare Fig. 5 and Figs. 9–11). At 42 (but not 28 or 36 days of gestation, GnRH-immunoreactive cells are detected in the epithelium of the medial olfactory pit, with N-CAM-immunoreactive axons on the nasal septum and in ganglia of the nervus terminalis, along with a greater number of N-CAM-immunoreactive cell bodies (Figs. 10–12). However, it's interesting to note that the majority, if not all, of the cells that traverse the cribriform plate and actually enter the medial basal forebrain with the central roots of the nervus terminalis are GnRH-immunoreactive (Boehm et al., 1994; and personal unpublished observations in human embryos). In some species, including the rat, the guinea pig, and the opossum, the GnRH immunoreactive cells are rarely seen in the epithelium of the olfactory pit (Schwanzel-Fukuda and Silverman, 1980; Schwanzel-Fukuda et al., 1985, 1988). Rather, the cords of cells emerge from the epithelium and begin to show GnRH-immunoreactivity on the nasal septum, or as they traverse the cribriform plate and enter the forebrain. It may be that some of the N-CAM-immunoreactive cells are destined to begin synthesis and secretion of GnRH on the nasal septum or as they traverse the cribriform plate and enter the brain with roots of the nervus terminalis (Schwanzel-Fukuda et al., 1996).

One notable difference seen in the 42- and 46-day-old human embryos compared to mice is the distinctive separation of the N-CAM immunoreactive cellulovascular bridge into lateral and medial nerve fascicles or ‘plexus-like laminae’ (Bossy, 1980) by primitive branches of the anterior cerebral arteries to the nasal regions, either side of midline (Fig. 11a, b). The lateral nerve fascicles or laminae are made up of the axons of the olfactory nerves and the Schwann (ensheathing) cells, which accompany them to the olfactory bulbs (Figs. 9a, b; 10a, b; 11a, b; and see Pearson, 1941). These cells are smaller and the axon

Fig. 8. (a) At 29–30 days of embryogenesis in an 8 μ m sagittal section of a human embryo, a larger, more elaborate vascular field is seen in the nasal mesenchyme (NM) between the olfactory pit (OP) and the developing forebrain (f). (b, c) At higher magnifications (compare with the same as seen in Fig. 7b and c) smaller, now diamond shaped N-CAM-immunoreactive cells are seen the epithelium of the olfactory placode (OP). In the vascular field, the blood vessels (bv) are larger and contain many more red blood cells (rbc). The N-CAM immunoreactive cells still aggregate around the blood vessels and at this age fine processes appear to sprout from these cells and form a kind of plexus in the nasal mesenchyme. Fig. 8c shows details of the association between the blood vessels and the N-CAM cell bodies and processes. No GnRH-immunoreactive cells are present in human embryos at these ages.



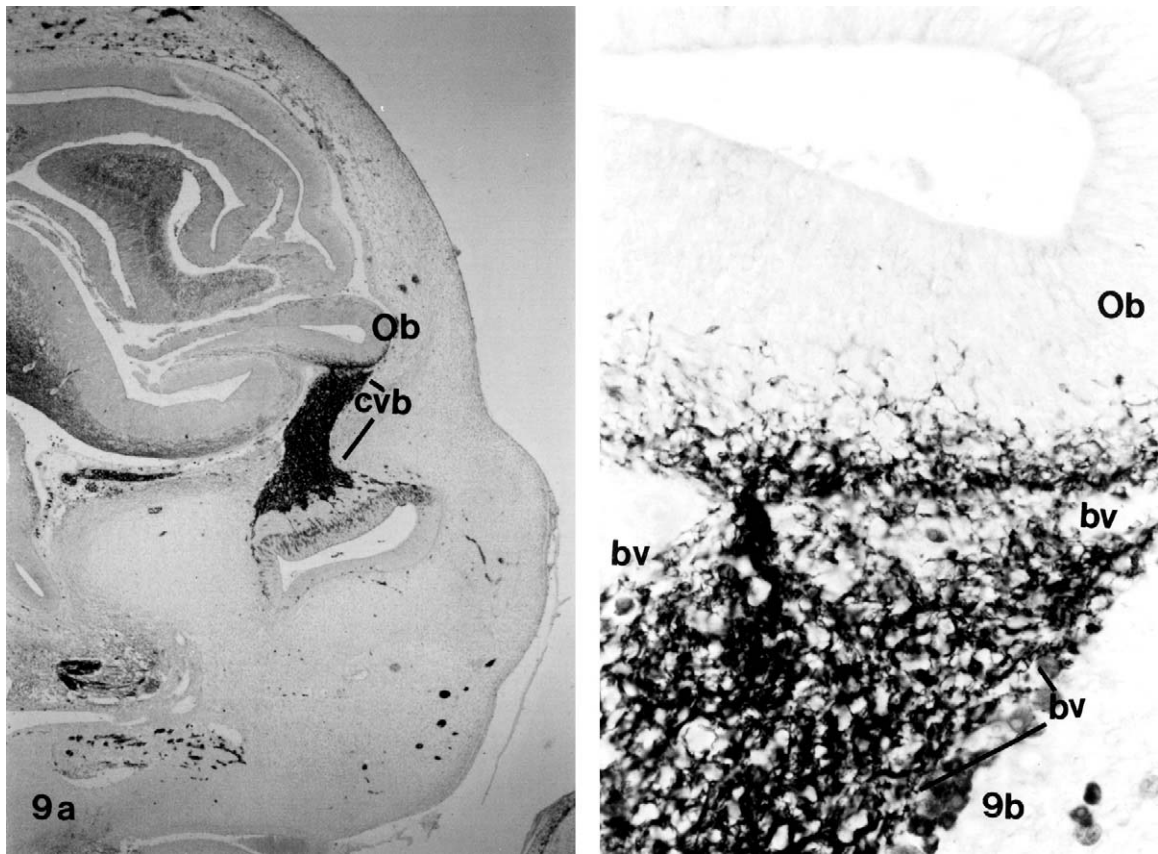


Fig. 9. (a) Low power photomicrograph of an 8 μ m sagittal section through a 42-day-old human embryo. A broad, thick band of N-CAM immunoreactive cell bodies and axons is seen in the more lateral parts of the cellulovascular bridge (cvb) and forms a distinctive, dense part of the migration route from the olfactory epithelium, through the nasal mesenchyme. The rostral part of this bridge is in contact with the developing olfactory bulb (Ob). These ingrowing axons are the central processes of the olfactory nerves, and they are accompanied by Schwann cells, which originate in the olfactory placode. These axons form an N-CAM-immunoreactive net-like cap over the anlage of the olfactory bulb. GnRH cells are never seen in these parts of the migration route. (b) At higher magnification, the contact of the N-CAM-immunoreactive axons can be seen in proximity to the surrounding blood vessels (bv).

bundles form a compact course from the epithelium of the olfactory pit into the developing olfactory nerve layer and the anlage of the olfactory bulb. The nerve fascicles or laminae from the medial part of the olfactory epithelium consist of the central processes of the vomeronasal and terminalis nerves (Pearson, 1941). In contrast to the lateral cellulovascular bridge and aggregate, the GnRH- and N-CAM-immunoreactive cells are seen on N-CAM-immunoreactive axons emerging into the nasal mesenchyme leading into the ganglia of the terminalis nerve along the medial part of the nasal septum (Figs. 3, 11a, b and 12). The GnRH and the N-CAM-immunoreactive cells are distinctly larger than the

N-CAM-immunoreactive Schwann cells seen among the axons of the olfactory nerves (Schwanzel-Fukuda et al., 1996).

The basement membrane along the ventral surface of the is breached in several places along the medial basal forebrain and cords of GnRH cells have begun pass into the brain (Fig. 11a, b). There is also evidence that capillaries have begun to vascularize the basal forebrain at these ages (Figs. 7a, b; 8a, b; 11a, b and 13a, b). By the end of the embryonic period, 57 postovulatory days or about 8 weeks, the human embryo is approximately 27–31 mm in length. At this time, the cranial nerves are visible and the choroid plexus is vascularized by deep branches of the an-

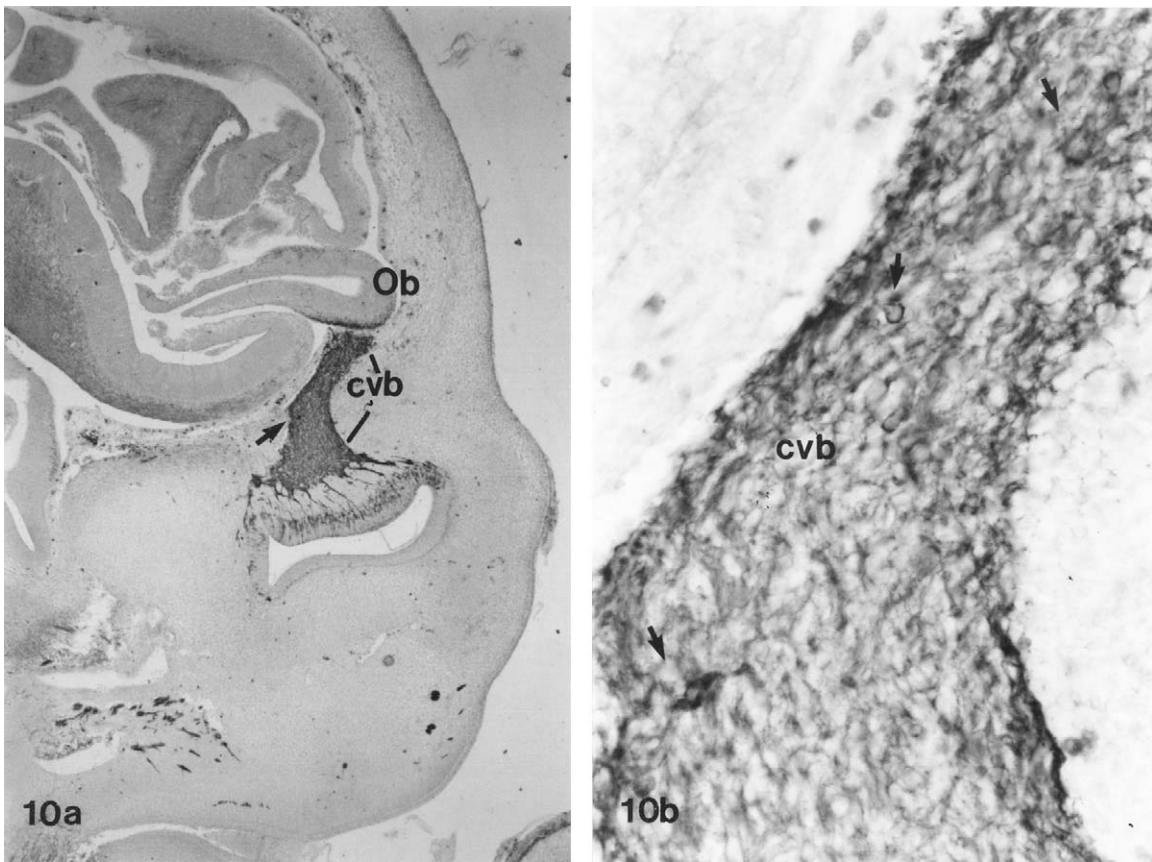


Fig. 10. (a) Low power photomicrograph of an 8 μ m sagittal section, medial to Fig. 9a and b, in the same 42-day-old human embryo. In this section, closer to midline, a few GnRH-immunoreactive cells (arrow) are seen among the N-CAM-immunoreactive cell bodies and axons of the cellulovascular bridge (cvb). In this section and in Fig. 9a and b, the connections between the olfactory placodal epithelium and the developing olfactory bulb can be seen clearly. (b) At higher magnification, the GnRH cells (arrows) can be seen along the migration route of the cellulovascular bridge. Please see this figure reproduced in the color plate, Fig. 16 (p. 76), for greater detail.

terior cerebral arteries (Theiler, 1989). The circulus arteriosus or 'circle of Willis' has been present on the ventral surface of the brain since approximately 37 postovulatory days and by 57 days closely resembles the adult pattern of vascularization (O'Rahilly and Muller, 1994).

The search for a single molecular or chemical cue which provides the magical 'Open Sesame' for the movement of GnRH-immunoreactive cells from the nose into the brain has thus far (at least at this writing) shown several promising yet still elusive clues. Schwarting and co-workers (Schwarting et al., 2001) showed that 'Deleted in colorectal cancer (DCC)' a vertebrate receptor for the guidance molecule netrin-1, during development of the olfac-

tory system is expressed in cells migrating from the olfactory epithelium and vomeronasal organ from embryonic days 11 to 14. They also found that DCC is 'downregulated' beginning at day 12 of embryogenesis. Study of GnRH cell migration in transgenic DCC $^{-/-}$ mice showed abnormal trajectories for the GnRH neurons, thus demonstrating that DCC is important in guiding the migration of the GnRH cells to their appropriate destinations. Susan Wray (Wray, 2001) discovered a novel factor which she termed 'nasal embryonic LHRH factor' or NELF which is expressed in both the peripheral and central nervous systems, including olfactory sensory cells and GnRH cells in nasal areas. Results of her study raised the possibility that NELF acts as a common guidance

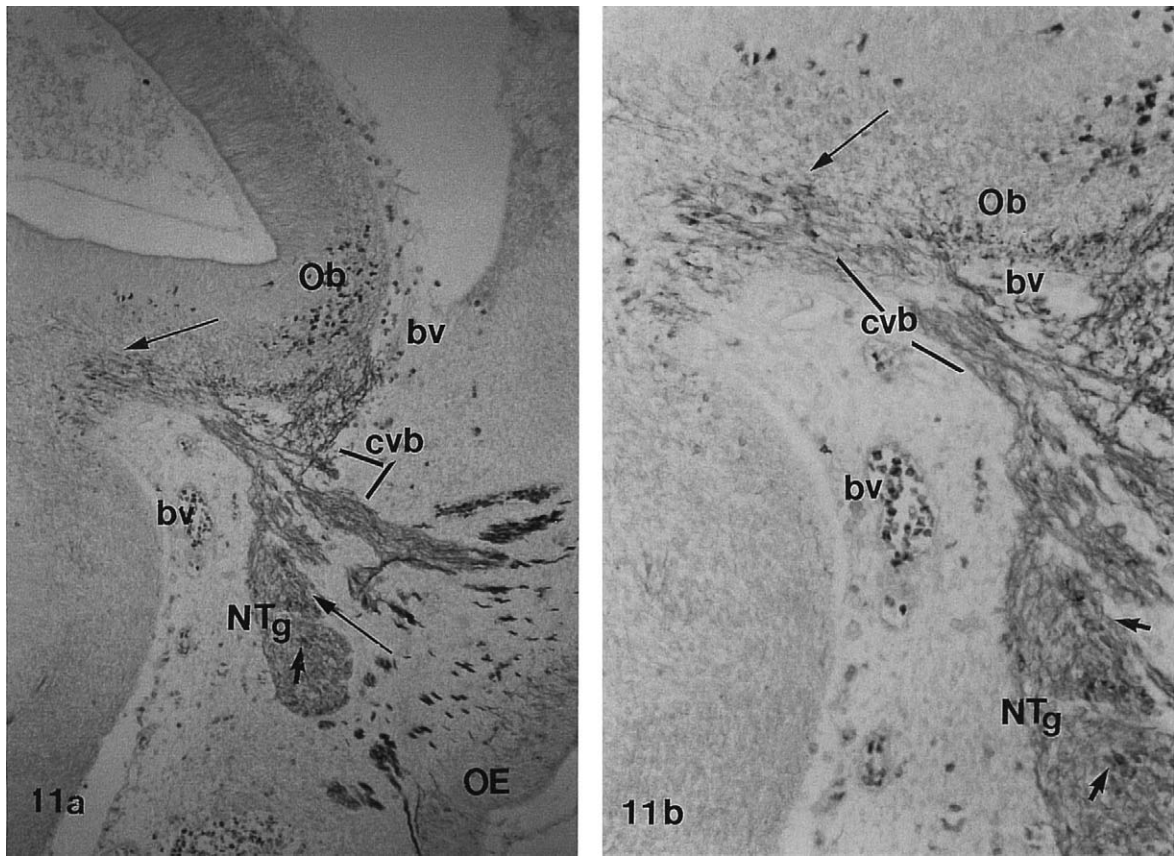


Fig. 11. (a) Low power photomicrograph of an 8 μ m sagittal section through the midline brain and nasal regions of a 46-day-old human embryo. In this section, a number of N-CAM-immunoreactive axons can be seen emerging from the olfactory epithelium (OE) onto the nasal septum and extending into contact with the rostral-ventral olfactory bulb. The ganglion terminale of the nervus terminalis (NTg) is also visible in this section, with its population of GnRH cells (arrow). (b) N-CAM immunoreactive axons accompanied by GnRH cells can be seen entering the ventral-medial forebrain, caudal to the medial surface of the olfactory bulbs. Note that the cellulovascular bridge (cvb) is in continuity with the basal forebrain at this point. The vascular layer (bv) which clearly separates the olfactory from the terminal-vomeronasal complex is visible in these near midline sections.

molecule for olfactory axon projections and the migration of GnRH cells, and thus acts as a migratory signal. All such studies show the importance, as anticipated, of signaling molecules, and suggest that the coordinated interactions of more than one factor may be essential for the complex migration of the GnRH cells into the brain. Part of this complexity may be due to the fact that GnRH is a crucial hormone for development of the reproductive system as well as for essential functions in puberty and adulthood (Silverman, 1988). The trajectories taken by the migrating GnRH cells and the eventual regulated release of GnRH may require a sequence of signals dependent on and changing with the stages of de-

velopment during the embryonic and fetal periods, both morphological and in response to circulating hormones.

Kallmann's syndrome: a genetic defect involving derivatives of the olfactory placode, including GnRH neurons

The genetic basis of Kallmann's syndrome was first described by Kallmann and co-workers (Kallmann et al., 1944). A similar disorder was described by De Morsier in his remarkable study of median cranioencephalic dysraphias and olfactogenital dysplasia (De Morsier, 1974). The puzzle of why this genetic de-

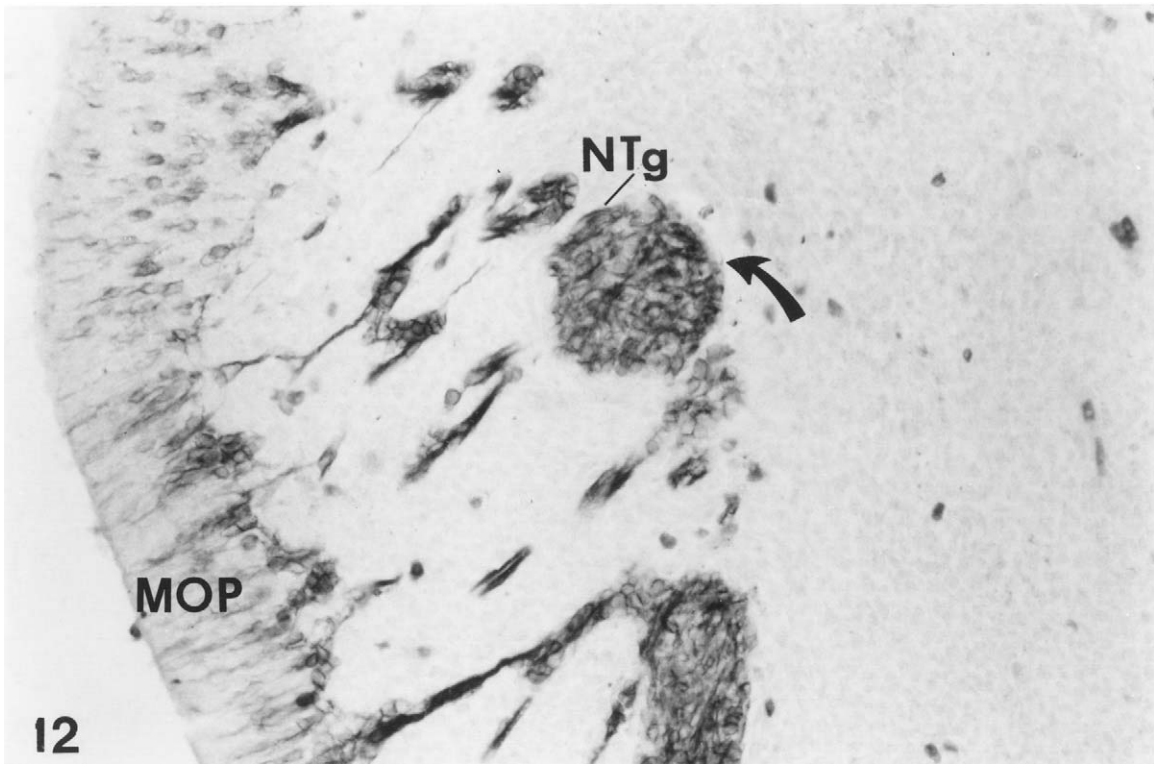


Fig. 12. An 8 μ m sagittal section near midline through the nasal mucosa showing the epithelium of the medial olfactory placode (MOP) and the ganglion terminale of the nervus terminalis (NTg). A population of GnRH-immunoreactive cells are visible among the N-CAM-immunoreactive cell bodies and axons in the smaller ganglia seen along the course of the nervus terminalis. Please see the color plate, Fig. 16 (p. 76), for greater detail.

fect involving olfactory placodal derivatives should become evident after they have undergone considerable differentiation is very likely worthy of study. Given our current hypothesis about the role of angiogenesis on development of the GnRH neuronal migration, we are looking back over our material to see if any clues can be found in the patterns of angiogenesis between the Kallmann fetus compared to those of the normal sex and age-matched controls.

In this laboratory, we have had the privilege of examining by immunocytochemical procedures and antibodies to GnRH, a 19-week-old male fetus with Kallmann's syndrome and three normal male fetuses of the same age (Schwanzel-Fukuda et al., 1989). The Kallmann fetus showed an absence of both olfactory bulbs, and no GnRH-immunoreactivity was located in any part of the brain. In striking contrast, thick fascicles of GnRH-immunoreactive axons and

clusters of GnRH neurons were seen deep in the nasal regions, on the nasal septum and along the dorsal surface of the cribriform plate (Fig. 14), and closely applied to either side of the crista galli, deep to the overlying meninges. In the nasal regions, the olfactory, the vomeronasal and the nervus terminalis fibers appeared to have developed normally from the olfactory placode, up to a point. The cribriform plate of the ethmoid bone showed eccentric perforations, and axons of the olfactory nerves ran through the perforations to the dorsal surface of the cribriform plate. Here, either side of midline, the olfactory nerve fibers formed neuromas, tangles of axons on the cribriform plate, deep to the meninges. Importantly, there was no indication of any contact with the forebrain. Deep in the nasal regions, the thick fascicles of GnRH axons and clumps of ganglion cells (Fig. 15) also enforced the impression that development of the vomeronasal and terminalis nerves,

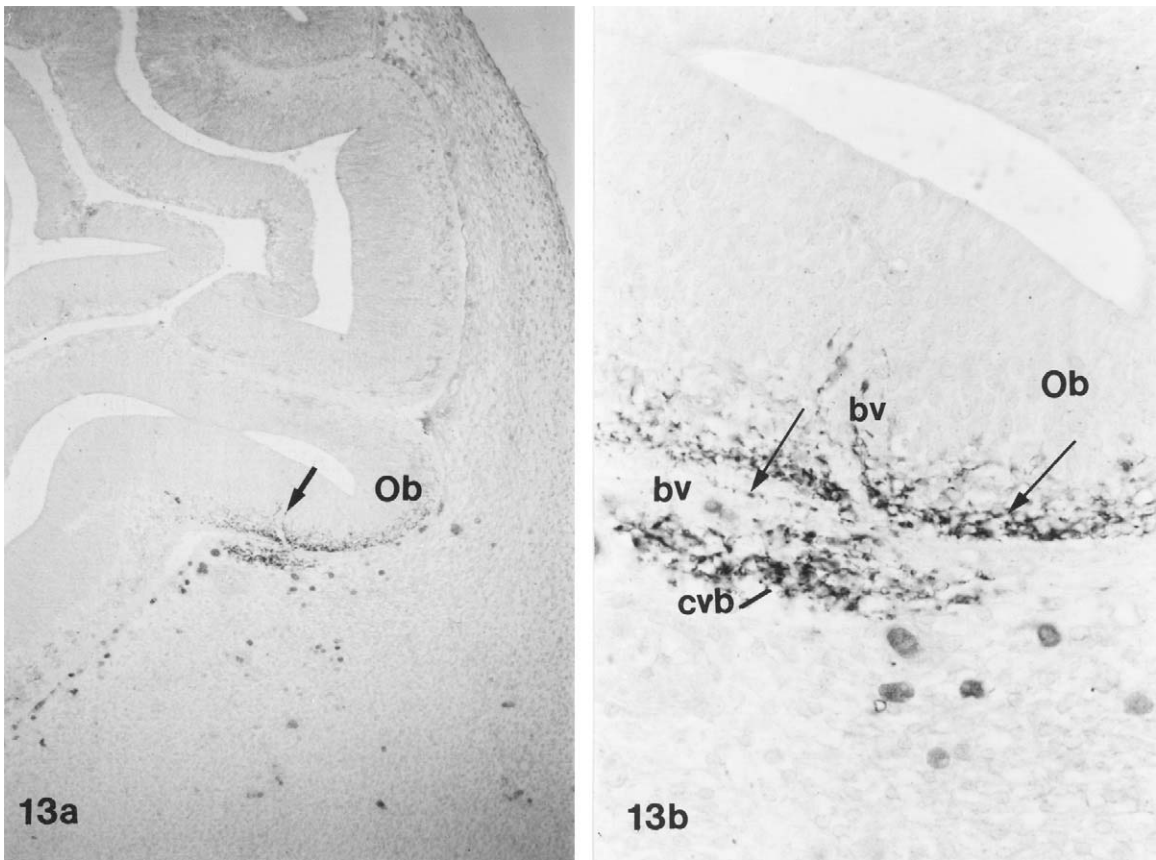


Fig. 13. (a) An 8 μ m sagittal section through the brain and nasal regions of a 42-day-old human embryo. N-CAM-immunoreactivity is seen in cells of the leptomeningeal vascular layer along the ventral surface of the developing brain (arrow), and in the walls of a small blood vessel (bv) which has broken through the basement membrane of the ventral surface of the olfactory bulb (OB). (b) At higher magnification a part of the cellulovascular bridge (cvb) and the pial vasculature (arrow) can be distinguished.

and the GnRH-immunoreactive neurons from the olfactory placode, was normal for an initial period of embryonic development.

Over the past months, we have examined Bouin's-fixed, paraffin embedded 8 μ m tissue sections of embryonic mice and humans, with antibodies to metalloproteinases (MMP-2) and to endothelial cell markers (Kalebic et al., 1983), including vascular endothelial growth factor (VEGF; Fig. 6a, b) and the tyrosine kinase receptors Flt-1 and Flk-1. Our purpose is to visualize and compare essential patterns in the development of the GnRH systems and the vasculature. One of the strongest coming from these observations is that during early embryogenesis, the areas of the brain undergoing rapid development with neurogenesis and the migration of cells through

a differentiating matrix have very high energy requirements for nutrients and disposal of waste products. Both sets of needs are best met by a rich blood supply.

For 13 years we have hoped to define the sequence of transient events (time-ordered and morphological) which result in the genesis of the GnRH neurons in the olfactory placode and the remarkable phenomenon of their migration along an elaborate 'cellulovascular bridge' from the placodal epithelium across the nasal septum and into the ventromedial forebrain. If the current hypothesis is correct, it will provide a large step toward understanding this intricate and transient system, repeated during embryogenesis in all mammals and most vertebrates.

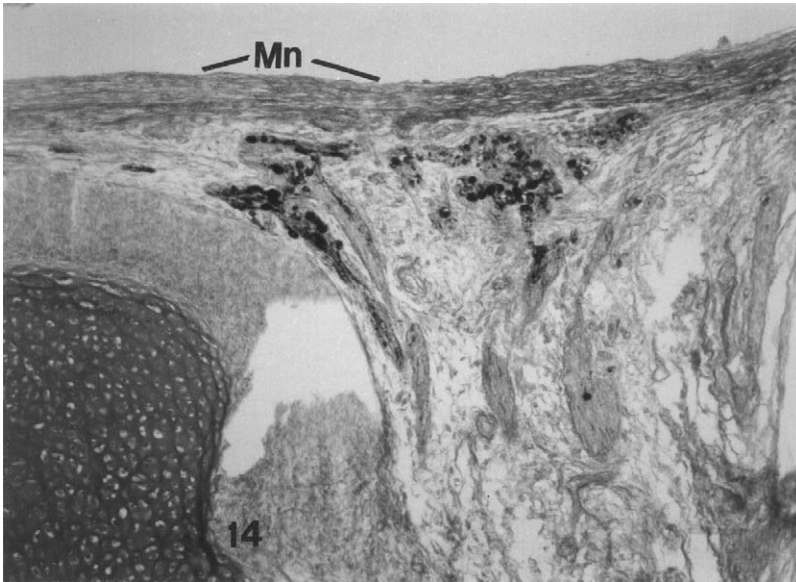


Fig. 14. Nineteen-week-old male fetus with Kallmann's syndrome. An 8 μ m sagittal section through the brain and nasal regions of this fetus shows an absence of the olfactory bulb, and an accumulation of GnRH immunoreactive ganglion cell bodies (arrow) and axons on the dorsal surface of the ethmoid bone, deep to meninges (Mn). No GnRH immunoreactivity was detected in any part of the brain. This section was counterstained lightly with cresyl violet.

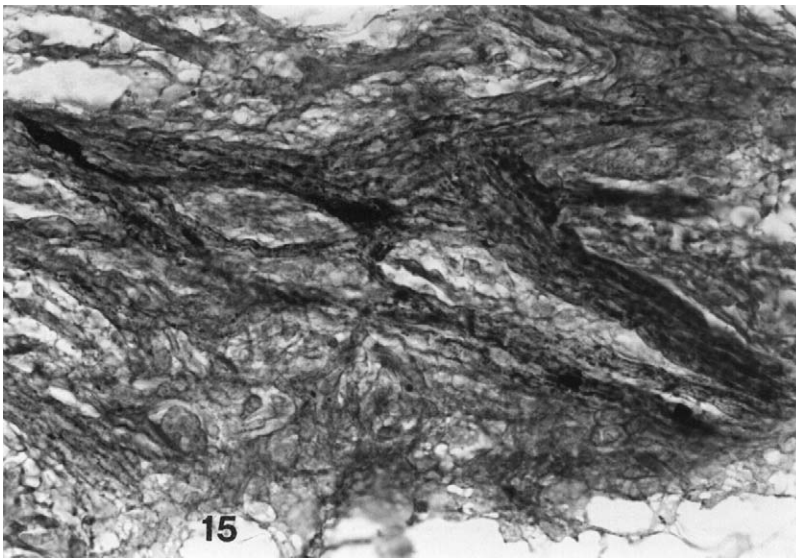


Fig. 15. Nineteen-week-old male fetus with Kallmann's syndrome. An 8 μ m sagittal section shows thick GnRH-immunoreactive axons bundles and cell bodies (arrows) in ganglia of the nervus terminalis in the nasal mucosa. Please see this figure reproduced in the color plate, Fig. 16 (p. 76).

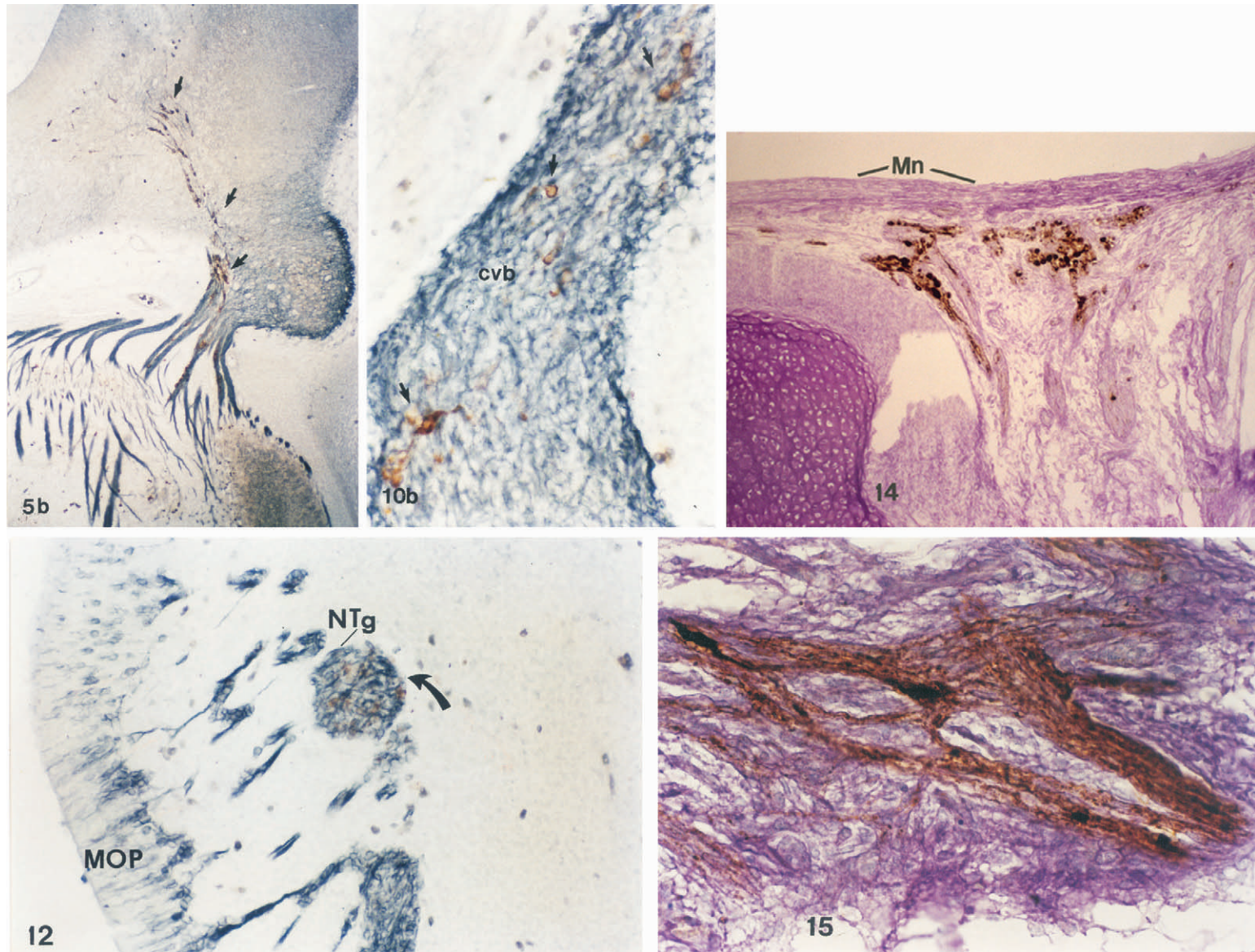


Fig. 16. Color plate to show the double-labeling of GnRH neurons and N-CAM-immunoreactive neurons seen in black and white in Figs. 5b, 10b, 12, 14 and 15. GnRH-immunoreactivity is seen as red-brown reaction product. N-CAM-immunoreactivity is seen as blue-gray reaction product. All sections are lightly counterstained with cresyl violet.

Acknowledgements

This research was supported by a Shannon award from the National Institutes of Health for M.S.-F. All studies in human embryos were carried out with the approval of the Internal Review Board of The Rockefeller University Hospital.

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CHAPTER 6

Recent advances in the pathogenesis of Kallmann's syndrome

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Introduction

Kallmann's syndrome (KS: olfactogenital dysplasia) represents the prototype of a human developmental defect caused by a disorder of axonal pathfinding and defective GnRH neuronal ontogeny. Affecting 1 in 8,000 males, and 1 in 40,000 females, olfacto-genital dysplasia is characterised by absence of olfaction (anosmia), due to olfactory bulb and tract agenesis and hypogonadotrophic hypogonadism (HH, the functional consequence of failed hypothalamo-hypophyseal gonadotrophin releasing hormone (GnRH) secretion. Although a largely sporadic condition, a significant proportion of KS is familial, with pedigrees demonstrating autosomal dominant, recessive and X-linked modes of inheritance (X-KS) (Oliveira et al., 2001; Quinton et al., 2001).

Genetics of KS

Distinct phenotypic differences exist between the autosomal and X-linked modes of transmission: In addition to anosmia and HH, X-KS is characterized by bimanual synkinesis (upper body mirror movements: 85%) and unilateral renal agenesis (33%). In autosomal KS, additional phenotypes include occasional midline developmental defects such as cleft palate,

choanal atresia, and coloboma (Oliveira et al., 2001; Quinton et al., 2001). Pedigree analysis in isolated XKS pedigrees, and positional cloning approaches led to the identification of the *KALI* gene in 1991 (OMIM: 308700). This encodes a 680 aa modular extracellular membrane associated protein (anosmin-1) comprising an N terminal whey acidic protein (WAP) like domain, followed by four consecutive fibronectin type III like repeats, and a histidine-rich C terminus. *KALI* is expressed in the mitral and tufted cells of the developing human and chick olfactory bulbs; these secrete anosmin-1 into the surrounding extracellular matrix suggesting a fundamental role in bulb histogenesis (Legouis et al., 1993; Duke et al., 1995; Hardelin et al., 1999). The additional autosomal genes, *KAL2* and *KAL3* (OMIM: 147950), have remained elusive because of infertility in patients, and therefore a lack of suitable pedigrees for study (MacColl et al., 2002).

Pathophysiology of KS

The pathophysiology of X-KS has been the most extensively studied hitherto. Endocrine profiling, histological/histopathological explorations and observations on comparative GnRH ontogeny, and finally, MRI imaging and electrophysiological investigations have given insights into the pathogenesis of this disease (Legouis et al., 1993; Kirk et al., 1994; Lutz et al., 1994; Duke et al., 1995, 1998; Schwanzel-Fukuda et al., 1996; Krams et al., 1997, 1999; Mayston et al., 1997; Quinton et al., 1997b; Hardelin et al., 1999; Schwanzel-Fukuda,

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1999; Deeb et al., 2001; Oliveira et al., 2001; Wray, 2001; Pitteloud et al., 2002).

Endocrine profiling demonstrates very low or absent gonadotrophin secretion in KS patients, but preserved pituitary responsiveness to exogenous GnRH, indicative of hypothalamic GnRH deficiency (Oliveira et al., 2001; Pitteloud et al., 2002), the consequence of a critical reduction in the 2,000 or so hypothalamic GnRH neurons, which normally discharge pulsatile GnRH into the median eminence capillary loops. Moreover, detailed immunohistochemical examination of a 19 week *KALI* deleted foetus revealed that GnRH neurons, and olfactory, n. terminalis and vomeronasal nerves axonal terminals (cranial nerve 1 complex) failed to reach the brain, instead terminating their journey from their olfactory placodal origin at the upper nasal sub-cribriform area. A pathogenetic cranial nerve 1 complex elongation pathway defect was suggested with, a consequent failure of olfactory bulb induction and ascending GnRH neuronal migratory arrest interpreted as representing a secondary event (Schwanzel-Fukuda, 1999). Postnatal nasal biopsies in X-KS demonstrate immature olfactory epithelial neuronal bodies – similar to appearances seen following experimental bullectomy (Schwob et al., 1993), and these, together with the identification of persistent GnRH neurons in olfactory epithelial biopsies confirm the normal ontogeny of the early olfactory placodal structures in X-KS (Quinton et al., 1997a).

GnRH neuronal ontogeny

GnRH neuronal ontogeny and its interrelationship with peripheral olfactory pathways is remarkably preserved across a broad range of vertebrate species, from: amphibians, (Muske and Moore, 1990), fish (Chiba et al., 1994) to chick, quail (Murakami et al., 1991), rats and primates (Schwanzel-Fukuda et al., 1996; Terasawa et al., 2001). Both GnRH and olfactory receptor neurons originate in the olfactory placodes, and olfactory bulb genesis is induced by pioneer olfactory axonal contact with telencephalic vesicles (Gong and Shipley, 1995). Disruption of this common pathway, not unexpectedly disturbs both olfactory and GnRH neuronal development. This is reinforced by brain MRI of patients confirming defective olfactory bulb formation and hypoplasia of

secondary processing areas including the entorhinal cortex (Quinton et al., 1996; MacColl et al., 2002).

XKS and synkinesis ('mirror movements')

Persistent mirror movements are seen in about 85% of X-KS patients. Focal magnetic stimulation of the motor cortex hand area in patients with synkinesis has revealed fast conducting bilateral corticospinal projections from each motor cortex, and cross correlation analysis of multi-unit EMGs recorded during simultaneous voluntary sustained activation of homologous left and right pairs of distal upper limb muscles confirms the presence of a common drive to left and right homologous motor neuron pools, demonstrating a novel ipsilateral corticospinal tract (Krams et al., 1997, 1999; Mayston et al., 1997). Statistical analysis of pooled white matter data from structural T1 weighted MR images using SPM-96 software in autosomal vs. X-linked KS patients also demonstrated a hypertrophied corticospinal tract in the X-KS cohort, consistent with the abnormal development of ipsilateral corticospinal tract fibres (Krams et al., 1999). Changes in regional cerebral blood flow with $H_2^{15}O$ -PET during an externally paced finger voluntary thumb/finger opposition task in the same individuals revealed ipsilateral M1 cortical activation in XKS supporting in addition the presence of an abnormal transcallosal pathway. However, it is also possible that sensory feedback from the involuntary mirroring hand may have contributed to these findings (Krams et al., 1997).

The above investigations suggest a defect in axonal guidance with aberrant decussation of the pyramidal corticospinal pathways KS – a hypothesis consistent with demonstration of the *KALI* transcript in the 45 post-fertilization developing human spinal cord (Duke et al., 1995).

In summary, loss of *KALI* function in X-KS leads to two separate neurological defects, in the developing olfactory system and in the descending corticospinal tracts. Both appear to be caused by axon targeting defects.

KAL1 and kidney development

Anosmin-1 demonstrates pleiotropism during embryonic development, and *KALI* and anosmin-1 are

also expressed during nephrogenesis at the ureteric bud/metanephric blastema interface, possibly participating in reciprocal mesenchymal–epithelial cell interactions necessary for organogenesis (Duke et al., 1998; Hardelin et al., 1999; Woolf, 2001). In 67% of males with X-KS, this defect is compensatable, with apparent preservation of normal renal function (Kirk et al., 1994); this is not invariant however, as we have recently provided evidence that the solitary kidney is frequently dysplastic leading to with proteinuria, hypertension (Duke et al., 1998) and early renal failure (Deeb et al., 2001). Adhesive interactions between the developing mesenchymal–epithelial cell boundaries are important for glomerular development and several of the missense mutations which cause X-KS alter the adhesion of kidney epithelial cells to an immobilised recombinant anosmin-1 (Soussi-Yanicostas et al., 1998; Robertson et al., 2001), supporting a putative ‘adhesive–inductive’ role for anosmin-1 in these interactions.

Cross-species ‘anosmins’

While cellular adhesion and axonal guidance appear to represent intrinsic properties of anosmin-1, akin to other molecules that influence development of the olfactory and GnRH systems, the developing corticospinal tracts and the kidneys (Dellovade et al., 1998a,b; Allen et al., 1999, 2000; Kramer and Wray, 2000; Schwarting et al., 2001; Woolf, 2001; Yokoyama et al., 2001) the absence of a murine model of KS has limited experimental progress in defining the biological actions of anosmin-1 and the characterisation of interacting ligands.

KAL-1 is of fundamental importance in the ontogeny of these systems in humans, reflected by the effects of loss of function mutations/deletions (Oliveira et al., 2001). *KAL-1* homologues have also been identified in *Drosophila* to *Caenorhabditis*, zebra fish, chick, and macaque (Schultz et al., 1998, 2000). All of these cross-species ‘anosmins’ contain a WAP domain, followed by one or more Fn3 domains—a domain combination that has been conserved during vertebrate and invertebrate evolution.

The *C. elegans* orthologue of *KAL-1* (*CeKal-1*) is expressed in a subset of neurons, as well as the excretory canal and uterine lumen (O. Hobert, pers. comm). Loss of function of the *CeKal1* generates a

number of low to medium penetrance phenotypes, including: ventral enclosure defects, ray (tail) abnormalities in males and neurite outgrowth/axon targeting defects (Rugarli et al., 2002). Neurite outgrowth defects include an extra branching dendrite in EF3 neurons that terminate prematurely and do not appear to make contact with their targets in male nematodes (Rugarli et al., 2002; Bulow personal communication). A similar effect is seen in nematodes overexpressing *CeKal1*, where neuron-specific overexpression induced dosage-dependent branching defects and axon misrouting (Bulow, personal communication). Studies in *C. elegans* also support a role for *KAL1* in cell adhesion, as a loss of function of *CeKal1* causes malformations in adherent junctions between epithelial cells leading to ventral enclosure defects (Rugarli et al., 2002).

What determines localisation of anosmin-1, and what genetic pathways influence its actions?

Heparan sulphate proteoglycans (HSPG) localise anosmin-1 to the surface of cells expressing protein (Soussi-Yanicostas et al., 1996) and binding of anosmin to heparan sulphate side chains are also important for its biological activity, as the phenotypic abnormalities resulting from overexpression of the *CeKal-1* homologue in *C. elegans* are nullified in genetic backgrounds deficient in heparan side chain synthesis and modification (Bulow, personal communication; Hobert, personal communication).

Molecular modelling studies of the four Fn3 domains from human anosmin-1 molecule reveal several basic regions, which may interact with heparan sulphate side chains (Robertson et al., 2001). Consistent with these observations, a deletion mutant of the Fn3 domains abolishes the biological activity of *CeKal1*, as does a point mutant in a region of the first Fn3 domain conserved between Human, Chick and *C. elegans* (Bulow et al., 2002). The physical interactions between anosmin-1 and heparan sulphate are clearly important, and currently being investigated in our laboratory.

Conclusion

In summary, at least three genes cause the X-linked and autosomal forms of KS. The study of X-KS pedi-

gresses indicate that loss of *KALI* function produces neurological defects caused by abnormal axonal targeting in the olfactory and corticospinal regions, and morphogenetic abnormalities in the developing kidneys. The conservation of domain structure in cross-species anosmins' indicate that protein function is also conserved at the molecular and cellular level. The *C. elegans* studies support this hypothesis, with loss of function and overexpression of mutants causing abnormalities in neurite outgrowth, axon targeting and cell-cell contacts during gastrulation/ventral enclosure. Future studies in our laboratory aim to investigate the function of *D. melanogaster* *Kal-1* (*DKal1*) by engineering a loss of function mutant, identify the specific sites of *DKal1* expression, overexpress the protein in these regions and identify interacting proteins. We believe this may subsequently enable us to identify interactants in the human genome data base thereby revealing potential autosomal loci for KS. Ultimately such genes would be validated as candidates for KS by mutational screening of autosomal KS pedigrees.

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SECTION II

GnRH receptors

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CHAPTER 7

Differences in structure–function relations between nonmammalian and mammalian GnRH receptors: what we have learnt from the African catfish GnRH receptor

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Introduction

Mammalian GnRH receptors differ from other G-protein coupled receptors (GPCRs) in lacking the intracellular C-terminal tail and in showing an exchange of two otherwise highly conserved Asp and Asn residues in TM 2 and 7, respectively. However, the first GnRH receptor characterized from a non-mammalian vertebrate, the African catfish, contains an intracellular C-terminal tail and has Asp residues in TM 2 and 7 (Fig. 1 and Tensen et al., 1997). Subsequently, the cloning of chicken (Troskie et al., 1997), goldfish (Illing et al., 1999), bullfrog (Wang et al., 2001), *Xenopus laevis* (Troskie et al., 2000), *Seriola dumerilii* (manuscript in preparation) and red seabream (manuscript in preparation) GnRH receptors revealed that all nonmammalian GnRH receptors have a C-terminal tail and the Asp/Asp motif in TM 2 and 7. Thus, these unique features described for mammalian GnRH receptors are not found in their nonmammalian counterparts.

Next to structural variations, differences between mammalian and nonmammalian GnRH receptors

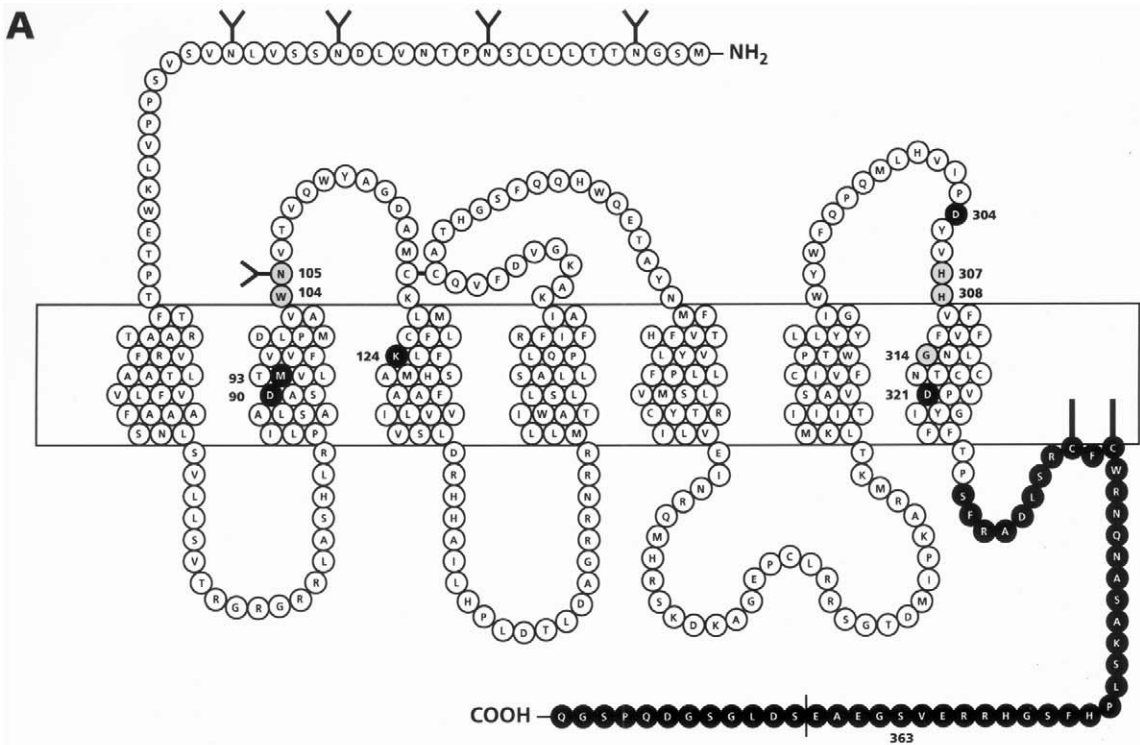
were found in their pharmacology and their regulation. In the following sections, the results of studies on catfish and mammalian GnRH receptor expression, regulation and activation, and ligand binding will be summarized. To this end, mammalian–nonmammalian chimeric GnRH receptors, site-directed mutagenesis of GnRH receptors, various GnRH analogs and a three-dimensional model of the receptor–ligand complex were used.

The carboxyl-terminal tail

In many GPCRs, the intracellular C-terminal tail has been demonstrated to be important for receptor expression (Iida-Klein et al., 1995; Oksche et al., 1998) and for regulatory processes, e.g. agonist-induced phosphorylation and subsequent receptor desensitization and internalization (Ferguson et al., 1996; Lefkowitz, 1998). The presence of the C-terminal tail in nonmammalian GnRH receptors enabled studies on the role of this C-terminal tail in these receptors as well as the functional consequences of its absence in mammalian GnRH receptors.

Progressive truncations of the C-terminal tail decreased cell surface expression of the catfish GnRH receptor (Blumenröhr et al., 1999), whereas the addition of the catfish C-terminal tail to the naturally tailless rat GnRH receptor resulted in elevated lev-

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	10	9	8	7	6	5	4	3	2	1
cfGnRH	G-NH ₂	P	N	L	G	H	S	W	H	pE
cGnRH-II	G-NH ₂	P	Y	W	G	H	S	W	H	pE
mGnRH	G-NH ₂	P	R	L	G	Y	S	W	H	pE

Diagram showing the interaction of GnRH analogs with a receptor. The receptor is represented by a cluster of seven circles labeled 1 through 7. The analogs are represented by dashed lines connecting to specific residues on the receptor: D304, G314, D321, K124, D90, and N105. The analogs are numbered 1 through 7.

els of receptor expression at the cell surface (Lin et al., 1998). The enhanced expression of mammalian GnRH receptors, fused to the C-terminal tail of the catfish GnRH receptor, in transfected cells could be of value in screening for therapeutically useful GnRH analogs. However, in mammalian gonadotropes, the expression level of the naturally tailless GnRH receptor seems to be sufficient, since it has been demonstrated that occupancy of 20% of GnRH receptors can evoke 80% of the biological response (Naor et al., 1980). In addition to enhancing mammalian GnRH receptor expression, the C-terminal tail of the catfish GnRH receptor has also been used as a linker between the rat GnRH receptor and the green fluorescence protein in order to visualize receptor localization in living cells (Cornea et al., 1999).

Furthermore, it has been demonstrated that the catfish GnRH receptor is susceptible to agonist-induced phosphorylation and that Ser³⁶³ in the C-terminal tail is the major phospho-acceptor site in this process (Blomenröhr et al., 1999). Mammalian GnRH receptors, on the contrary, are resistant to agonist-dependent phosphorylation due to the lack of a C-terminal tail (Willars et al., 1999). This was substantiated by comparing wild-type mammalian GnRH receptors with chimeras of the mammalian GnRH receptor fused to the C-terminal tail of either the mammalian TRH receptor or the catfish GnRH receptor: only the latter two are phosphorylated in an agonist-dependent manner (Willars et al., 1999). Thus, the presence of a functional C-terminal tail confers agonist-induced GnRH receptor phosphorylation.

Agonist-induced phosphorylation seems to be a prerequisite for desensitization of the catfish GnRH receptor. It undergoes rapid desensitization of the IP response in HEK 293 and COS-7 cells (Heding et al., 1998; Willars et al., 1999), whereas the tailless rat GnRH receptor showed no desensitization of the IP response within seconds to minutes in α T3-1, HEK 293 or COS-7 cells (Anderson et al., 1995; Heding et al., 1998; Willars et al., 1998, 1999). Addition of the C-terminal tail of either the catfish GnRH receptor or the rat TRH receptor to the rat GnRH receptor resulted in rapid desensitization of the IP response (Heding et al., 1998; Willars et al., 1999). Possibly the pattern of GnRH release demands GnRH receptors that are susceptible to desensitization in the catfish pituitary, where GnRH is locally secreted in the vicinity of the gonadotropes, whereas in mammals GnRH is released into the portal system in a pulsatile fashion. Still, mammalian gonadotropin secretion is desensitized upon continuous GnRH stimulation, because the absence of mammalian GnRH receptor desensitization is compensated by a desensitizable postreceptor mechanism, i.e. the down regulation of IP₃ receptors and consequent desensitization of GnRH effects on cytosolic calcium (McArdle et al., 1999). On the other hand, it has not been investigated properly whether catfish gonadotropes show rapid desensitization of the gonadotropin secretion because catfish GnRH receptors are susceptible to desensitization within seconds to minutes. It has been demonstrated that continuous administration of 10 μ M mGnRH analog to perfused catfish pituitaries resulted in a constant amount of released gonadotropins measured

Fig. 1. Schematic side view of the catfish GnRH receptor in the plasma membrane (A). Black circles indicate residues that have been investigated by site-directed mutagenesis, grey circles indicate residues that have been identified by three-dimensional molecular models for the binding of cGnRH-II or cGnRH-II-R8 to the catfish GnRH receptor. Schematic top view of the catfish GnRH receptor as seen from the extracellular side with each helix represented by a circle and the extracellular interconnecting loops (ELs) and the N-terminal domain as solid lines (B). The helices are organized sequentially in a counterclockwise fashion. Extracellular loops 1 and 2 are joined by a disulfide bridge. D³²¹ (TM 7) is supposed to be protonated, thereby allowing an interaction with D⁹⁰ in TM 2. Moreover, K¹²⁴ and G³¹⁴ interact with each other in a way that TM 3 and 7 come into close proximity. M⁹³ (TM 2) sterically hinders an interaction of D⁹⁰ (TM 2) and K¹²⁴ (TM 3). N¹⁰⁵ (EL 1) interacts with the carboxyl-terminal G¹⁰-NH₂, K¹²⁴ (TM 3) with the amino-terminal pE¹, and D³⁰⁴ (EL 3) with R⁸ of mGnRH as well as with Y⁸ of cGnRH-II. The aromatic ring of Y⁸ of cGnRH-II is embedded in a pocket formed by the aromatic side chains of residues W¹⁰⁴, H¹⁰⁷ and H¹⁰⁸ in the receptor. Agonist binding to the receptor triggers phosphorylation of S³⁶³ in the intracellular carboxyl-terminal tail. S³⁶³ and the last twelve amino acid residues of the C-terminal tail are important for β -arrestin-dependent receptor internalization. In addition, the C-terminal tail plays a role in cell surface expression of the receptor and ligand binding.

in 10-min interval fractions (De Leeuw et al., 1986). This finding might indicate that catfish gonadotropes are not susceptible to desensitization. However, rapid desensitization occurs within seconds to minutes and a study monitoring gonadotropin secretion during the first 10 minutes has never been performed on catfish gonadotropes.

The process of agonist-induced internalization of the catfish GnRH receptor also depends on receptor phosphorylation, since the removal of the phosphorylation site resulted in impaired receptor internalization compared to the wild-type receptor (Blomenröhr et al., 1999). However, truncated catfish GnRH receptors, still containing the phosphorylation site, also showed slower internalization kinetics than the wild-type receptor. The last 12 amino acids of the C-terminal tail seemed to be important for interaction with accessory proteins like β -arrestin and as such influence agonist-dependent receptor internalization (Blomenröhr et al., 1999). The rat GnRH receptor, on the contrary, has been demonstrated to internalize in a β -arrestin-independent, but dynamin-dependent, manner (Vrecl et al., 1998; Heding et al., 2000). Rat GnRH receptor-internalization kinetics are slower than those of the rat TRH receptor and of the catfish GnRH receptor, but an enhanced internalization rate was achieved under conditions of high β -arrestin when the rat TRH receptor C-terminal tail was added to the rat GnRH receptor (Heding et al., 1998). The β -arrestin-independent part of the catfish GnRH receptor internalization is similar to that of the rat GnRH receptor (Willars et al., 1999). Apart from the C-terminal tail, that is important for β -arrestin-dependent internalization, other residues in the catfish GnRH receptor protein may be involved in the β -arrestin-independent part of receptor internalization, since studies on mutant mammalian GnRH receptors have provided evidence for the importance in the internalization of the mammalian GnRH receptor of conserved amino acids in the DRY/S triplet in the second intracellular loop, a conserved apolar amino acid in the third intracellular loop, and of aromatic amino acids in TM 7 (Arora et al., 1995, 1996, 1997; Chung et al., 1999). Homologous residues in the catfish GnRH receptor may also be involved in the β -arrestin-independent part of catfish GnRH receptor internalization.

Residues in TM 2 and TM 7

The apparent interchange of otherwise highly conserved Asp and Asn residues in TM 2 and 7 of the mammalian GnRH receptors raised the possibility that these two residues interact with each other bringing TM 2 and 7 into close proximity. Studies on mutant mouse GnRH receptors indeed indicated a functional and spatial relationship of these two side chains (Zhou et al., 1994). Data from all other GPCRs studied are in accordance with a spatial proximity of the conserved TM 2 and TM 7 side chains (reviewed in Flanagan et al., 1999). The nonmammalian GnRH receptors, however, have two Asp residues at the homologous positions (Fig. 1 and Tensen et al., 1997). Possibly, the presence of the two Asp residues in nonmammalian GnRH receptors represents an evolutionary intermediate between the conserved Asp/Asn arrangement found in most GPCRs and the Asn/Asp motif of mammalian GnRH receptors. Indeed, crystallographic studies have demonstrated that Asp side chains can occur in spatial proximity within proteins and can form hydrogen bonds when one of the Asp side chains is protonated (Fig. 1) (Davies, 1990; Harrison and Weber, 1994).

It has been described that Asp⁹⁰ in TM 2 of the catfish GnRH receptor is implicated in receptor protein expression at the cell surface (Blomenröhr et al., 2001). This is in accordance with findings on the mouse GnRH receptor that also requires the Asn residue at the homologous position in TM 2 for receptor expression (Flanagan et al., 1999). Furthermore, it has been demonstrated for the catfish GnRH receptor that a negatively charged residue in this part of TM 2 is important for ligand binding and signaling (Blomenröhr et al., 2001). It is more likely that Asp⁹⁰ is necessary for a proper receptor conformation than for direct interaction with the ligand. Based on a model for the human GnRH receptor it was speculated that Asp⁹⁰ in TM 2 interacts with Lys¹²⁴ in TM 3 and as such stabilizes the inactive state of the catfish GnRH receptor. However, the role of these two residues in catfish GnRH receptor binding and activation is rather independent (Blomenröhr et al., 2001).

The acidic side chain of Asp³²¹ in TM 7 does not seem to play a role in either ligand binding or

efficient receptor coupling to G proteins (Blomenröhr et al., 1997). The homologous Asp³¹⁸ residue in the mouse GnRH receptor, on the contrary, has been implicated in efficient PLC coupling (Flanagan et al., 1999). Mammalian GnRH receptors have obviously fixed the requirement for an acidic side chain in TM 7, in contrast to most other GPCRs that require an acidic side chain in TM 2 for receptor activation (Flanagan et al., 1999).

In conclusion, it seems that the acidic side chain of Asp⁹⁰ in TM 2 of the catfish GnRH receptor is important for catfish receptor functioning, whereas Asp³²¹ in TM 7 is likely to be protonated. As such, TM 2 and TM 7 of the catfish GnRH receptor are capable of coming into close proximity, as has been postulated for mammalian GnRH receptors as well as for other GPCRs.

Receptor–ligand interactions

Mammalian GnRH receptors have a high affinity for GnRH peptides with a positively charged Arg on position 8 (Millar et al., 1989). It has been demonstrated that the negatively charged Glu³⁰¹ of the mouse GnRH receptor plays a role in the recognition of Arg⁸ in the ligand (Flanagan et al., 1994). The catfish GnRH receptor contains an Asp residue at the position homologous to Glu³⁰¹ in the mouse GnRH receptor (Tensen et al., 1997). However, this nonmammalian GnRH receptor does not show a high affinity for mGnRH that contains an Arg on position 8 (Blomenröhr et al., 1997). To our surprise, mutagenesis studies and a three-dimensional molecular model of the catfish GnRH receptor having cGnRH-II-R8 docked in the binding pocket revealed that Asp³⁰⁴ of the catfish GnRH receptor nevertheless recognizes Arg⁸ in mGnRH and in chimeric GnRH analogs (cfGnRH-R8, cGnRH-II-R8, cGnRH-II-dW6,R8; Blomenröhr et al., 2002; Fig. 1). It is likely that the low affinity of the catfish GnRH receptor for mGnRH is rather due to an unfavorable fit of residue 5 (Tyr) and/or residue 7 (Leu) of this ligand into the binding pocket of the catfish GnRH receptor than to the inability of this receptor to specifically recognize Arg⁸ in mGnRH.

Instead of a preference for mGnRH, the catfish GnRH receptor, like other nonmammalian GnRH receptors, shows a high affinity for cGnRH-II (Seal-

fon et al., 1997). Using native and chimeric GnRH analogs varying at positions 5, 7 and 8, we identified His⁵, Trp⁷ and Tyr⁸ as features of cGnRH-II conferring specificity for the catfish GnRH receptor (Blomenröhr et al., 2002). Results of binding studies with the native and chimeric peptides on D304A and D304N mutant catfish GnRH receptors suggested that Asp³⁰⁴ is not important for the recognition of Tyr⁸ in cGnRH-II (Blomenröhr et al., 2002). However, the molecular model for binding of cGnRH-II to the catfish GnRH receptor indicated that Tyr⁸ is able to hydrogen bond with Asp³⁰⁴ in the wild-type receptor (Blomenröhr et al., 2002; Fig. 1). But in the absence of Asp³⁰⁴ Tyr⁸ can also make a hydrogen bond with Ala¹⁹⁵, and the aromatic ring of Tyr⁸ is embedded in a pocket formed by the aromatic rings of residues Trp¹⁰⁴ in TM 2 and His³⁰⁷ and His³⁰⁸ in TM 7 (Blomenröhr et al., 2002; Fig. 1). Therefore Asp³⁰⁴ is less important for the recognition of Tyr⁸ in cGnRH-II than of Arg⁸ in mGnRH.

Molecular dynamics simulations further illustrate that His⁵ of cGnRH-II is in close proximity with Gln²⁹⁵ and His³⁰⁷ of the catfish GnRH receptor (Blomenröhr et al., 2002), whereas Tyr⁵ of mGnRH interacts with Asp²⁹³ (homologous to Gln²⁹⁵ of the catfish GnRH receptor) in the human GnRH receptor (Hoffmann et al., 2000). The Trp⁷ residue of cGnRH-II is embedded in a hydrophobic pocket formed by residues of TM 3, 4 and 7 together with residues of the second extracellular loop (Blomenröhr et al., 2002).

In addition, binding studies on a Lys¹²⁴-mutant catfish GnRH receptor (Blomenröhr et al., 2001) and molecular models for the binding of cGnRH-II to the catfish GnRH receptor (Blomenröhr et al., 2002) implicated Lys¹²⁴ (TM 3) as contact site for the amino-terminal pGlu¹ and Asn¹⁰⁵ (EL 1) for the carboxyl-terminal Gly¹⁰-NH₂ (Fig. 1). Moreover, cGnRH-II binds to the catfish GnRH receptor in a constrained β -turn conformation, with Gly⁵ facing to the entrance of the binding pocket (Blomenröhr et al., 2002). These results are in accordance with findings on mGnRH binding to mammalian GnRH receptors (Zhou et al., 1995; Davidson et al., 1996; Hoffmann et al., 2000).

Conclusion

The studies on the catfish GnRH receptor are an example as how comparative studies between evolutionary distant animal species may reveal similarities and differences in molecular forms of hormones and their cognate receptors, and of mechanisms of action. That is important to understand the functioning of hormone-controlled processes and to formulate general principles.

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CHAPTER 8

Regulation of GnRH and its receptor in a teleost, red seabream

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Introduction

As well as in mammalian species, gonadotropin-releasing hormone (GnRH) is well known as a primary factor which stimulates the synthesis and release of gonadotropin (GTH) from the pituitary in teleosts (see reviews by Peter, 1983; Okuzawa and Kobayashi, 1999). In these two decades, studies on GnRH of teleosts have significantly progressed since Sherwood et al. (1983) discovered a teleost specific GnRH, namely salmon GnRH (sGnRH); molecular forms and localizations of GnRHs in teleosts brain have been intensely studied (see reviews by Amano et al., 1997; Sherwood et al., 1997; Okuzawa and Kobayashi, 1999). However, the regulatory mechanism of the synthesis and release of GnRH in teleosts are not well understood compared to mammals. Therefore, we aimed to reveal the mechanisms underlying the regulation of the synthesis and release of GnRH, in order to help solve several basic and prac-

tical problems in fish reproductive physiology and aquaculture, such as the control of puberty in fish.

Red seabream, *Pagrus (Chrysophrys) major*, belongs to the order perciformes, the largest and evolutionally the most advanced fish group. It spawns almost everyday even in captivity during its spawning season, thus red seabream is an excellent experimental model for the reproductive biology of fish. In addition, it is one of the most important species for aquaculture in Japan. In this chapter, we will describe our recent achievements in studies on GnRH and GnRH receptor (GnRH-R) in red seabream, and propose possible models for the regulatory mechanisms of the onset of puberty and the seasonal reproduction of this species.

Molecular forms of GnRH and distribution in brain

To date, 14 different forms of GnRH have been identified based on the primary structure or complementary DNA (cDNA) in vertebrates (Carolsfeld et al., 2000; Okubo et al., 2000a; Yoo et al., 2000; Montaner et al., 2001; Adams et al., 2002). Among the 14 forms, 11 GnRHs and 8 GnRHs have been detected in fish and teleosts, respectively. In teleosts, two or three molecular forms of GnRH were found

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in the brain of all species examined, with chicken (c) GnRH-II as a common form (Okuzawa and Kobayashi, 1999). Combined studies using high-performance liquid chromatography (HPLC) and radioimmunoassay (RIA) revealed that three GnRHs; namely, sGnRH, cGnRH-II, and seabream (sb) GnRH exist in the brain of red seabream (Fig. 1). Molecular cloning of cDNAs encoding corresponding GnRHs (Okuzawa et al., 1994a, 1997; Kumakura et al., unpublished results) confirmed the existence of these three forms in the red seabream brain. On the other hand, two GnRHs, namely sbGnRH and sGnRH have been detected in the pituitary of red seabream by HPLC/RIA study (Mori et al., unpublished results). Quantification of GnRH with specific RIA revealed that the concentration of sbGnRH in the pituitary is about 2,000 fold higher than sGnRH (Senthilkumaran et al., 1999), suggesting that sbGnRH is more important in terms of GTH secretion from the pituitary.

We have studied the localization of the three GnRHs in the brain of red seabream with immunocytochemistry and *in situ* hybridization (Okuzawa et al., 1997; Okuzawa et al., unpublished results), and found three distinct GnRH neuronal systems in the brain of red seabream, namely the terminal nerve (TN), preoptic, and midbrain GnRH systems, producing sGnRH, sbGnRH, and cGnRH-II, respectively (Fig. 2). The sbGnRH-producing cell bodies in the preoptic area (POA) project their axons mainly to the pituitary, while sGnRH- and cGnRH-II-producing cells project their axons widely in the brain, but not to the pituitary. These morphological characteristics of the GnRH neuronal systems are common to other perciform and pleuronectiform fish species, such as the African cichlid, *Haplochromis burtoni* (White et al., 1995), dwarf gourami, *Colisa lalia* (Yamamoto et al., 1995), tilapia, *Oreochromis mossambicus* (Parhar, 1997), gilthead seabream, *Sparus aurata* (Gothilf et al., 1996), European sea bass, *Dicentrarchus labrax* (Gonzalez-Martinez et al., 2001) and barfin flounder, *Verasper moseri* (Amano et al., 2002a). These results clearly indicate that sbGnRH produced in the preoptic GnRH neuronal system functions as hypophysiotropic form of GnRH (GnRH in its true meaning). While sGnRH and cGnRH-II have neuromodulator or neurotransmitter functions as suggested by Oka (1997).

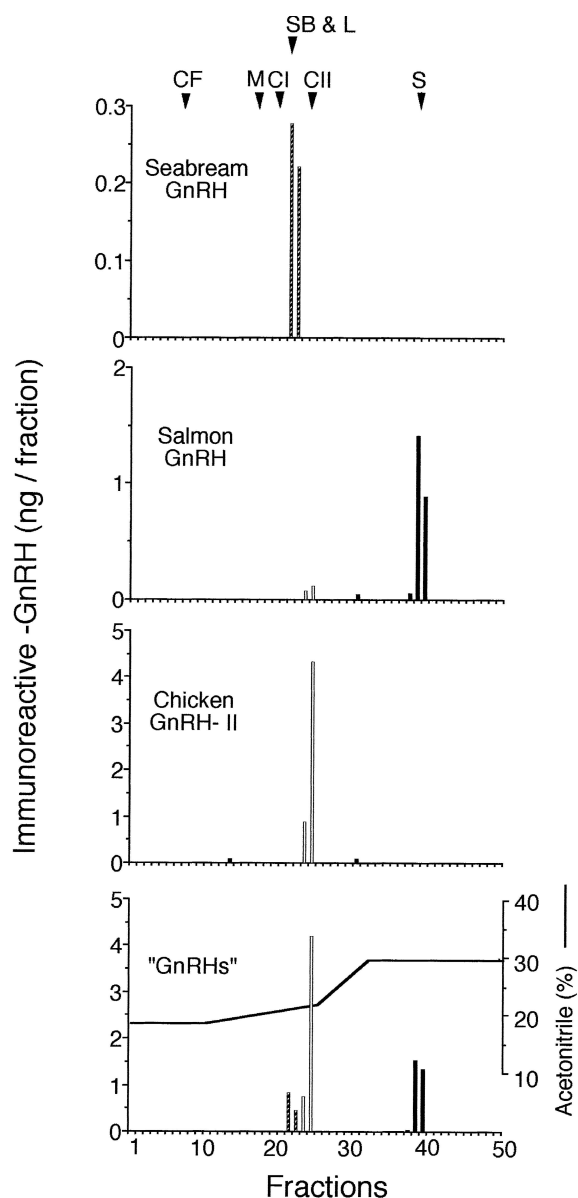


Fig. 1. Identification of molecular species of GnRH contained in the brain of red seabream, *Pagrus major*, by a combined study of high performance liquid chromatography (HPLC) and radioimmunoassays (RIAs). Extract of brains was fractioned by reverse phase HPLC, and each fraction was tested by four kinds of RIA, namely three specific RIAs that are specific to seabream GnRH, salmon GnRH and chicken GnRH-II, and a RIA that can detect several GnRH molecules ('GnRHs'). The arrow heads indicate retention times of synthetic GnRH peptides, CF, catfish GnRH; M, mammalian GnRH; CI, chicken GnRH-I; SB, seabream GnRH; L, lamprey GnRH-I; CII, chicken GnRH-II; S, salmon GnRH.

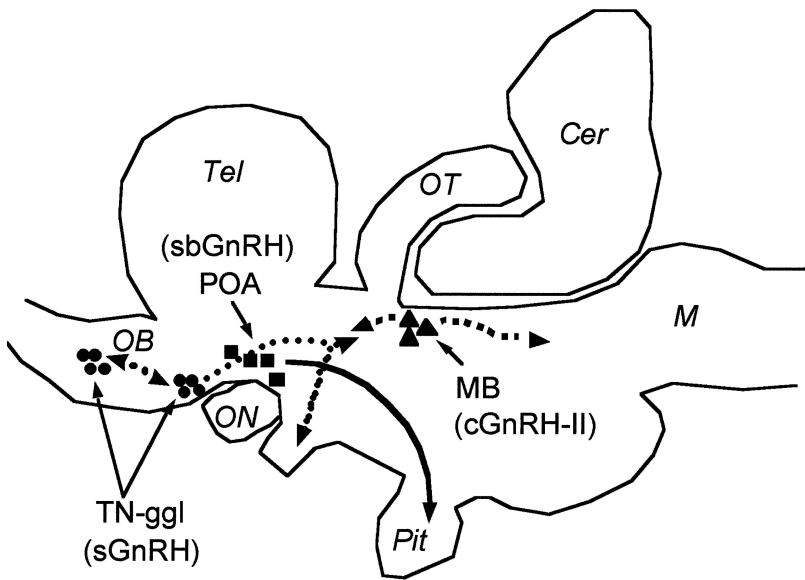


Fig. 2. Schematic illustration of a para-sagittal section of the red seabream, *Pagrus major*, showing the distribution of cell bodies of salmon (s) GnRH (circles), seabream (sb) GnRH (squares) and chicken (c) GnRH-II (triangles), and the projections (arrows). Cer, cerebellum; M, medulla oblongata; MB, midbrain; OB, olfactory bulb; ON, optic nerve; OT, optic tectum; Pit, pituitary; POA, preoptic area; Tel, telencephalon; TN-ggl, ganglions of the terminal nerve. Modified from Okuzawa et al. (1997).

Development of GnRH neuronal systems

At the present time it is well established that GnRH neurons originate in the olfactory organ, and then migrate into the forebrain during the development in mammals, birds and amphibians (see Chapter 3 by Murakami et al. in this volume). Also in teleosts, several reports (Chiba et al., 1994; Parhar et al., 1995; Parhar, 1997; White and Fernald, 1998) have demonstrated that terminal nerve GnRH neurons are derived from the olfactory placode. However, the origin of the three distinct GnRH systems in advanced teleosts, such as perciform fish is still uncertain. We, therefore, studied development of the three distinct GnRH neuronal systems in red seabream by immunocytochemistry and *in situ* hybridization (Ookura et al., 1999). sGnRH immunoreactive (ir) cells were first detected one day after hatching (day 1) in the olfactory pit. Then, sGnRH-ir cells disappeared from the olfactory organ, and they were observed along the olfactory nerve and in the telencephalon (Fig. 3A). cGnRH-II-ir axons and cell bodies were first observed in the midbrain tegmentum on day 3 and day 6, respectively, and the number

of cell bodies and fibers increased with development (Fig. 3B). Immunoreactivity to sbGnRH first emerged in cell bodies in the POA, in axons elongated from the POA to the pituitary, and in the neurohypophysis around day 37 (Fig. 3C). These results suggest that sGnRH, sbGnRH, and cGnRH-II systems have distinct ontogenetic origins, namely the olfactory placode, POA, and midbrain, respectively. Parhar (1997) reported similar results for tilapia. On the other hand, in a perciform African cichlid, it is demonstrated that both sGnRH and sbGnRH neurons migrate from the olfactory placode to their appropriate adult location in the brain, namely the ganglion of the terminal nerve and the POA, respectively, whereas cGnRH-II neurons arise directly from the midbrain ventricle (White and Fernald, 1998). Recently, Amano and co-workers conducted olfactory epithelia lesions with fry of masu salmon, *Oncorhynchus masou*, just after hatching. Fish were sampled 212 days after the operation, and it was found that neurons expressing sGnRH mRNA (a pivotal hypophysiotropic form in salmonoids) were detected in the ventral telencephalon and POA in the control group while there were no such neurons

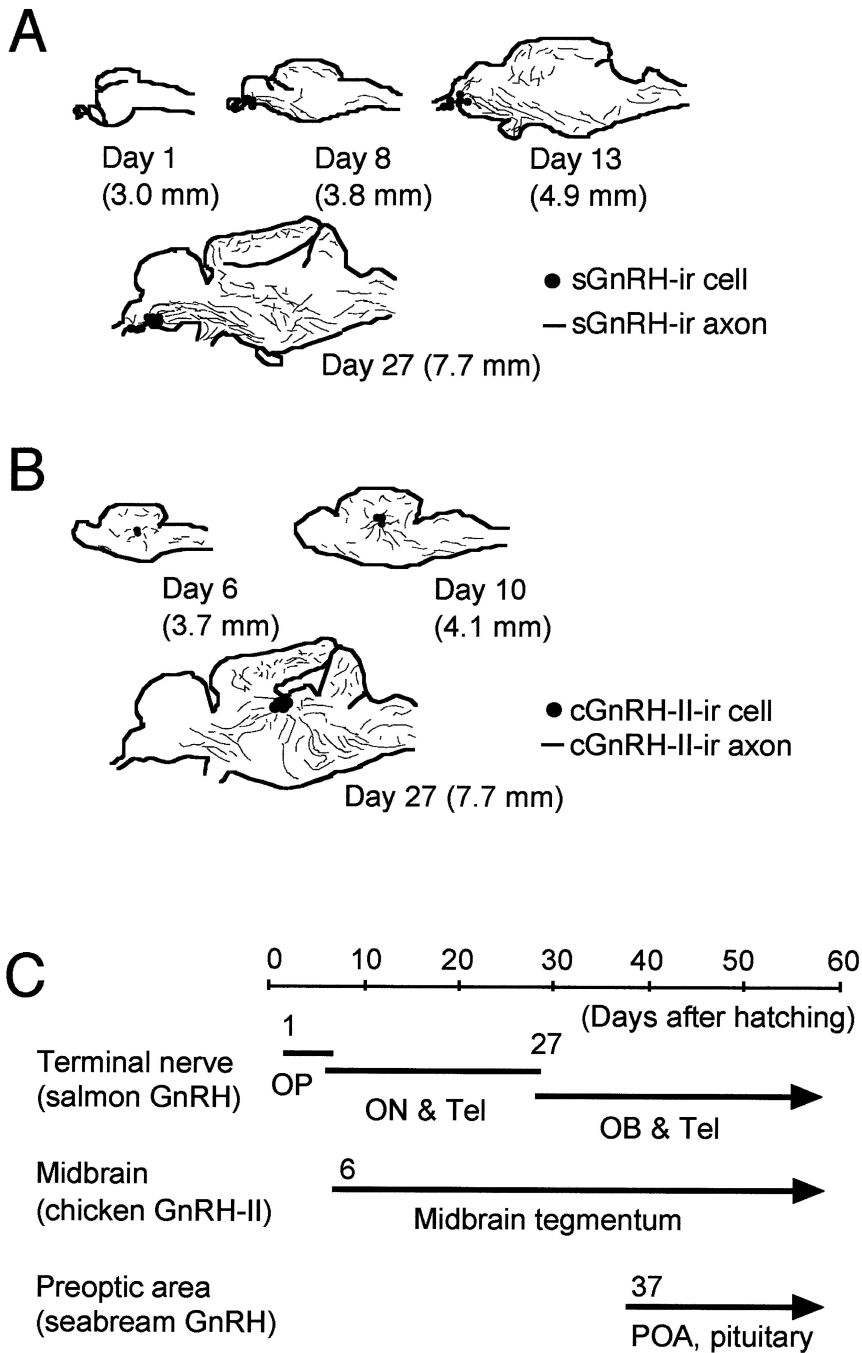


Fig. 3. Schematic illustrations of sagittal sections of red seabream, *Pagrus major*, showing the ontogeny of salmon (s) GnRH (A) and chicken (c) GnRH-II (B) neurons, and a summary of the ontogeny of the three GnRH neuronal systems in red seabream (C). Number in parentheses indicates the body length of each specimen. OB, olfactory bulb; ON, olfactory nerve; OP, olfactory pit; POA, preoptic area; Tel, telencephalon.

in those areas in the olfactory epithelium lesioned group (Amano et al., 2002b). This clearly indicates that the origin of the sGnRH neurons in the ventral telencephalon and POA in masu salmon is the olfactory epithelium. A similar experimental approach is necessary to conclude the precise origin of sbGnRH neurons in POA in perciforms, despite it being very difficult because of the tiny size of the fry of perciform fish compared to salmonids.

Seasonal changes in GnRH and GnRH mRNA

Two serious difficulties in the quantification of GnRH peptides or mRNAs occur; one is the multiplicity of GnRH molecular species within the brain of one organism, and the other is the multiple functions of one specific GnRH molecule in distinct neuronal systems (see Okuzawa and Kobayashi, 1999). It is necessary to employ the appropriate quantitative method specific for each GnRH peptide or mRNA species, and to distinguish the same GnRH molecular species belonging to the distinct GnRH neuronal systems in one organism, for example sGnRH in TN and preoptic GnRH neuronal systems in salmonid and cyprinid fish. In these fishes, histological techniques, such as immunocytochemistry or *in situ* hybridization, are the only way to overcome the problem so far, and dividing the brain into several parts is not appropriate. In this section, I will refer to our recent work on red seabream and recent studies in which the two above mentioned difficulties were overcome.

Red seabream is a seasonal spawner; in female red seabream vitellogenesis commences in February and the ovaries attain full maturity in April. Their spawning season begins in April and continues until May, and fish spawn almost every day during the spawning season. A seasonal change in the gonadosomatic index (GSI, gonad weight/body weight \times 100) of 2-year-old female red seabream is shown in Fig. 4. Fish were immature and mean GSI was low in October. They had ovaries containing oocytes at the perinucleolus stage. In December, the mean GSI slightly increased and oocytes at the oil stage were observed in their ovaries. In February, the mean GSI further increased, and the most advanced oocytes attained the primary yolk globule stage. In March, the GSI increased rapidly, and the stage of the oocytes proceeded to the secondary yolk globule stage. In

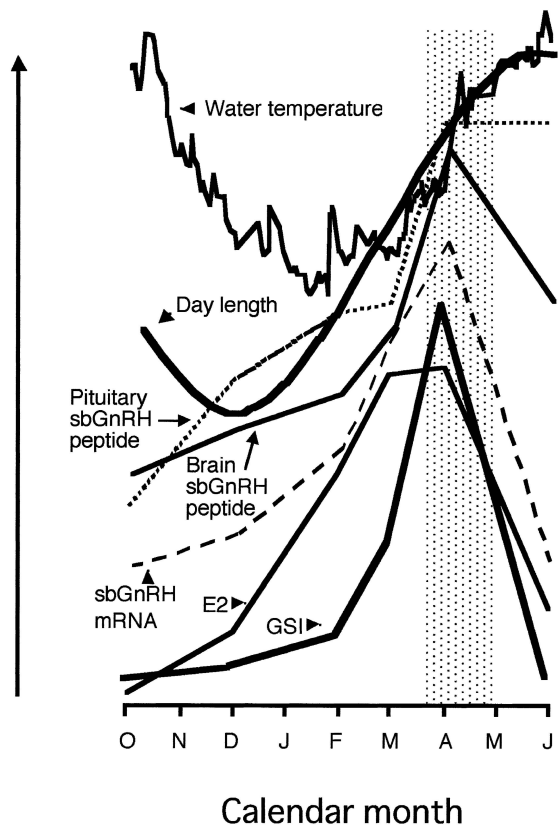


Fig. 4. Schematic illustration of the seasonal fluctuations of environmental factors (water temperature and day length), and gonadosomatic index (GSI), serum levels of estradiol-17 β (E₂), brain and pituitary levels of seabream (sb) GnRH peptide and brain levels of sbGnRH mRNA in female red seabream, *Pagrus major*. The shaded area indicates the spawning season. Modified from Senthilkumaran et al., 1999 and Okuzawa et al., unpublished results.

April, the spawning season, the mean GSI reached the maximum and all sampled fish had oocytes at various developmental stages from the perinucleolus to the mature stage. In June, after spawning season, the GSI decreased abruptly. Ovaries of all fish were regressed and only oocytes at the perinucleolus stage were observed in the ovaries. Seasonal fluctuations of serum sex steroids, estradiol-17 β (E₂) (Fig. 4) and testosterone (T) (data not shown) were closely associated with changes in GSI.

In order to obtain basic information for understanding the regulatory mechanism of GnRH, we have studied the seasonal changes in the three native GnRHs (sGnRH, cGnRH-II and sbGnRH) in the

brain and pituitary using a specific RIA for each form of GnRH (Senthilkumaran et al., 1999), and the levels of messenger RNA (mRNA) encoding the three GnRHs of female red seabream (Okuzawa et al., unpublished results) using ribonuclease (RNase) protection assay (Fig. 4). The three GnRH neuronal systems in red seabream have distinct localizations and functions, and the three distinct GnRH molecular species are produced in different GnRH neuronal systems (Fig. 2), therefore we can observe fluctuations of activity of each GnRH neuronal system, even if we use the whole brain sample for estimation of GnRH peptide or mRNA.

Both brain and pituitary sbGnRH levels increased from October (immature phase) and reached peaks in April (spawning phase) in correlation with the increase in GSI and vitellogenesis. Brain levels of sbGnRH decreased in June (regressed phase) but the levels were still rather high and similar to levels in March (vitellogenic phase). However, the pituitary levels of sbGnRH remained high in completely regressed fish in June. On the other hand, levels of sGnRH and cGnRH-II in the brain were high in immature (October) and regressed (June) phases, but remained low during the spawning phase (Senthilkumaran et al., 1999). Pituitary levels of sGnRH were much lower than those of sbGnRH and showed no seasonal change, and pituitary cGnRH-II was always below the limit of detection (Senthilkumaran et al., 1999).

The levels of sbGnRH mRNA were correlated with the ovarian development (Fig. 4); the levels of sbGnRH mRNA of immature fish in October and December were low, and increased in February and March in association with active vitellogenesis. The sbGnRH mRNA levels reached maximum levels in April, and after which they rapidly decreased with the ovarian regression in June. In contrast, the levels of sGnRH mRNA showed no variation throughout the year and those of cGnRH-II mRNA displayed only slightly elevated levels in March and April (data not shown). Taken together, the levels of both brain sbGnRH peptide and mRNA were associated with the gonadal status, while seasonal fluctuations of peptides and mRNAs of the other two GnRH forms were rather independent of the gonadal status. Although several reports about the changes in sbGnRH have been published after the discovery

of this third form of GnRH in advanced teleost (Powell et al., 1994), to our knowledge, our work on red seabream is the only example that shows a clear correlation between the brain GnRH peptide or mRNA contents and gonadal maturation. Therefore, together with the morphological observations based on immunocytochemistry and *in situ* hybridization analyses mentioned in the former section (Fig. 2), it is strongly suggested that sbGnRH is the pivotal hypophysiotropic form of GnRH in red seabream. To sum up the evidence showing that sbGnRH is the 'real' GnRH in red seabream: (1) sbGnRH is dominant in the pituitary; (2) solely sbGnRH neurons in the POA project to the pituitary; (3) only sbGnRH peptide in the brain and pituitary increased with gonadal maturation; (4) only sbGnRH mRNA in the brain increased with the gonadal maturation, and decreased with the gonadal regression.

In many studies, the pituitary content of GnRH peptide has been shown to be associated with gonadal maturation; sGnRH in the pituitary increased in parallel with gonadal maturation in rainbow trout, *Oncorhynchus mykiss* (Okuzawa et al., 1990) and masu salmon (Amano et al., 1992, 1993). In a sex changing fish, gilthead seabream (*Sparus aurata*), mature males and females had higher sbGnRH concentrations in the pituitary than bisexual recrudescence fish (Holland et al., 1998). sbGnRH and cGnRH-II levels in the pituitary of maturing striped bass correlated to changes in oocyte diameter and GSI (Holland et al., 2001). In a pleuronectiform fish turbot, *Scophthalmus maximus*, sbGnRH content in pituitary increased in association with the increase in oocyte diameter (Andersson et al., 2001). In our experiments, although sbGnRH levels in the pituitary were correlated with gonadal maturation, the levels remained high in completely regressed fish in June. Similar results were reported in turbot (Andersson et al., 2001) and a viviparous teleost, the grass rockfish (*Sebastes rastrelliger*) (Collins et al., 2001); in post-spawning turbot sbGnRH content in pituitary was high and comparable to mature fish. In grass rockfish, sbGnRH content in brain and pituitary were markedly higher in post-spawn females and regressed males than in other reproductive conditions. Although Collins et al. (2001) explained this phenomenon as a result of a decline in the sbGnRH secretion at the end of reproductive cycle, further

studies with different species of teleosts, including viviparous species, are required to explain the physiological meaning of the high sbGnRH levels in the pituitary of regressed fish.

Although the level of mRNA is considered to be a good indicator of the biosynthesis of peptides, only a few studies have been performed about GnRH mRNA levels in teleosts. Amano et al. (1995a) reported that sGnRH mRNA expression in the ventral telencephalon and POA of the brain, detected by *in situ* hybridization, significantly increased during gonadal maturation in masu salmon. Gothilf et al. (1997) reported the preovulatory changes in the levels of three GnRH (sGnRH, sbGnRH and cGnRH-II) mRNAs in female gilthead seabream using RNase protection assay, and found that all three mRNAs fluctuate throughout the day, reaching highest levels 8 h before spawning, concurrent with the preovulatory GTH-II (LH) preovulatory surge. This suggests the involvement of the three GnRHs in the control of ovulation and spawning. Further studies of GnRH mRNA levels will elucidate the mechanism controlling the annual fluctuations of the activities in GnRH neuronal systems.

Regulation of GnRH activities

Regulation of GnRH neuronal activities in teleosts was clearly documented by Yu et al. (1997), therefore in this section, we will concentrate on our recent study in red seabream and recent studies of other investigators. Red seabream is a good experimental model for the study of the regulation of the GnRH neuronal system, because the sbGnRH system of red seabream is localized only in POA and functions only as a hypophysiotropic hormone (namely, GnRH in its true meaning) and does not function as a neurotransmitter within the brain. We will discuss the effect of environmental and physiological factors on the activities of preoptic sbGnRH system.

Environmental factors

It is well accepted that environmental factors, such as photoperiod and water temperature, strongly affect seasonal reproductive cycles in many teleosts (see review by Lam, 1983). We consider that red seabream, a seasonal spawner is not an exception;

it is very likely that photoperiod and/or water temperature are critical factors for the control of annual reproductive cycle of this species as well as other spring spawners such as cyprinid fishes (Hanyu et al., 1983).

The abrupt increase in brain sbGnRH peptide and mRNA levels of female red seabream from February to April (Fig. 4) is closely correlated with the rapid increase in day length at the same period (Fig. 4), thus we can hypothesized that the long photoperiod up-regulates the sbGnRH synthesis and in turn enhances GTH secretion from pituitary and gonadal development in red seabream. To test this hypothesis, we have conducted preliminary experiments about the effect of photoperiod on sbGnRH mRNA expression of female red seabream in November, and found no difference in both the gonadal development and the sbGnRH mRNA levels in brain between long and short photoperiod groups (Okuzawa et al., unpublished result). Therefore, photoperiod seems not to be an important factor for gonadal maturation of red seabream at least in females in this season. However, further experiments, for example, conducted in other seasons, are required to conclude that photoperiod is not important. There is no report concerning the relationship between photoperiod and GnRH synthesis except studies on masu salmon (Amano et al., 1995b, 1999), a typical autumn (short photoperiod) spawner, similar to other salmonid fishes. Immature male masu salmon were reared under long (16 hours L: 8 hours D) or short photoperiod (8 hours L: 16 hours D) regimes. After two months, precocious maturation occurred and the number of sGnRH mRNA expressing cells in the ventral telencephalon and POA, determined by *in situ* hybridization, had increased in the short photoperiod group, while fish remained immature and the number of sGnRH expressing cells was unchanged in the long photoperiod group. Furthermore, in castrated masu salmon (Amano et al., 1999), the number of sGnRH mRNA-expressing cells in POA had increased under short photoperiod but not under long photoperiod. Therefore, photoperiod affects sGnRH synthesis in the brain of masu salmon, and this effect is not mediated by gonadal factors such as sex steroids.

Another environmental factor for the reproduction of red sea bream is water temperature. Most likely, similar to spring spawning cyprinids, such as bit-

tering, *Acheilognathus tabira* (Shimizu and Hanyu, 1982) and honmoroko, *Gnathopogon caerulescens* (Okuzawa et al., 1989), increase of water temperature (above 20°C) in late May may inactivate the hypothalamo–hypophysial–gonadal axis, maybe through down-regulation of sbGnRH synthesis, and in turn terminates its spawning season. Probably, high water temperature may keep the red seabream reproductively inactive during summer season. An artificial abrupt decrease of water temperature to the optimum water temperature (from 28°C to 18°C) combined with a long photoperiod (15 hours L: 9 hours D) induced gonadal maturation and spawning in winter, four months earlier than the natural spawning season in red seabream (Kato, personal communication). Therefore, initiation factor for gonadal recrudescence in red seabream is considered to be a decrease of the water temperature to optimum. On the other hand, an increase of water temperature to optimum in combination with serum steroid levels (see below) in spring may function as an accelerator of sbGnRH synthesis (Fig. 4) and in turn pituitary and gonadal activities in red seabream. Red seabream, kept in a higher temperature (16°C) than the ambient water temperature during winter, initiated spawning 20 days earlier than the natural population (Fukusho et al., 1986). To our knowledge, reports on the effect of water temperature on GnRH activities are very scarce. Okuzawa et al. (1994b) reported that the optimum (15°C) water temperature increased sGnRH content in the brain and pituitary of a cyprinid fish honmoroko. This study, however, did not distinguish sGnRH in the preoptic system from sGnRH in the terminal nerve system, which is not essential for gonadal maturation or spawning of cyprinid (Kobayashi et al., 1992, 1994). Further studies are required to elucidate the effect of water temperature on GnRH activities.

Social interactions also affect the activity of the preoptic GnRH neurons; in territorial males of the African cichlid, which are characterized as aggressive and reproductively active, have significantly larger hypothalamic GnRH neurons than non-territorial males. Furthermore, a switch in the social status of an adult male causes a corresponding change in the GnRH neuron size (Francis et al., 1993). Similar situations have been described in a labrid fish, bluehead wrasse (*Thalassoma bifasc-*

iatum); terminal phase males have 2–3-fold more GnRH preoptic cells than primary phase males and females (Grober and Bass, 1991). Quantification of brain GnRH peptide and/or GnRH mRNA will enable a clearer picture to be drawn of the regulation of GnRH by social cues.

Steroids

Besides the environmental factors mentioned above, physiological or internal factors are also important. Amongst physiological factors, gonadal steroids are one of the most probable candidates for the regulator of GnRH neuronal system in teleosts. There are several reports indicating that sex steroids affect the GnRH system in the brain, especially articles on positive feedback in male fish are common. In male and female bluehead wrasse, a sex changing fish, implants of 11-ketotestosterone (11KT), the major androgen of teleosts, induced increases in the GnRH preoptic cell number (Grober et al., 1991). In male masu salmon, administration of 17 α -methyltestosterone increased the number of sGnRH-expressing neurons in POA (Amano et al., 1994). In male African catfish, *Clarias gariepinus*, T and E₂ exerted a positive influence on the amount of catfish GnRH (Dubois et al., 1998, 2001). In male tilapia, *Oreochromis niloticus*, E₂ increased the number of preoptic sbGnRH neurons (Parhar et al., 2000). On the other hand, studies in female fish are rather scarce. In female eels, *Anguilla anguilla*, E₂ induced an increase in the levels of brain and pituitary mammalian GnRH, which is the preoptic GnRH of eels (Montero et al., 1995).

In female red seabream, seasonal fluctuations of the levels of E₂ (Fig. 4) and T (data not shown) in serum were parallel to those of the sbGnRH mRNA levels, suggesting that sex steroids affect sbGnRH synthesis via a positive feedback mechanism. To examine this hypothesis, we have conducted several experiments of *in vivo* steroid implants using silastic capsules. Prepubertal (16-months-old) female red seabream were implanted with silastic capsules containing either E₂, 11KT, T, or a solvent (control) in May, and reared under natural environmental conditions for three weeks. Implantation of 11KT increased the brain sbGnRH mRNA levels, estimated by RNase protection assay, while E₂ and T had no

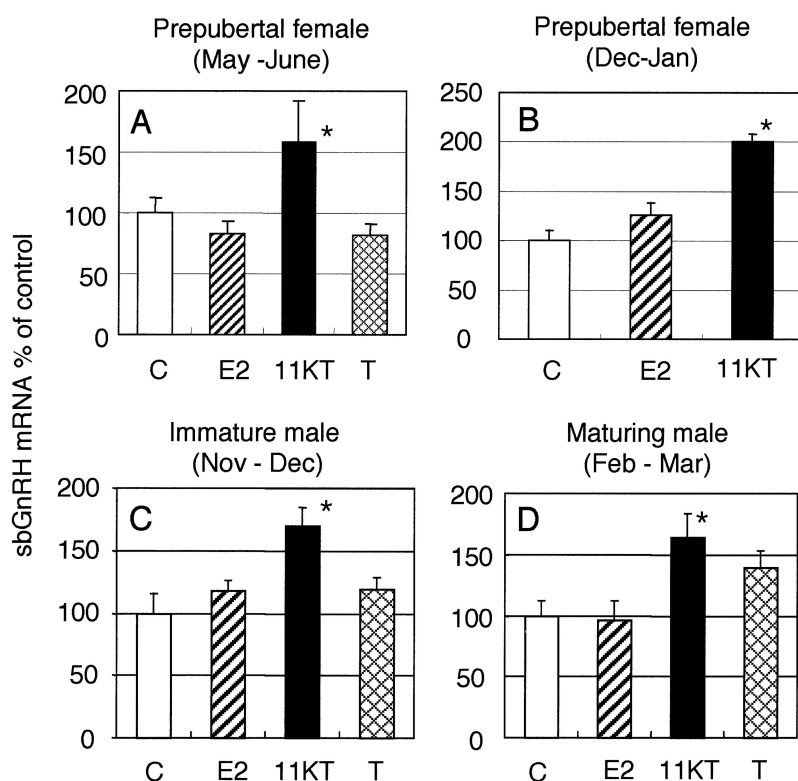


Fig. 5. Effect of a one month implantation of sex steroids on seabream (sb) GnRH messenger RNA levels in the brains of female and male red seabream, *Pagrus major*. C, control; E2, estradiol-17 β ; 11KT, 11-ketotestosterone; T, testosterone. Bars represent mean \pm SEM of 4–6 fish. Asterisks indicate significant differences compared with the control ($P < 0.05$; One-way ANOVA after logarithmic transformation and Duncan's multiple range test).

effect (Fig. 5A). We obtained similar results in another experiment, conducted in December, using the same age females or hermaphrodite red seabream (Fig. 5B). In addition, female red seabream which were artificially matured by the GnRH agonist exhibited no increase in brain sbGnRH mRNA levels despite both serum E₂ and T concentrations being markedly elevated (Kumakura et al., unpublished results). These data suggest that in prepubertal female red seabream E₂ and T, two major sex steroids of females, do not affect the brain sbGnRH mRNA expression, and that 11KT which is contained in female red seabream serum with a low concentration (less than 100 pg/ml, Yamaguchi et al., unpublished results) exerts a positive effect. The physiological role of the positive feedback effect of 11KT on the brain sbGnRH neuronal system in females is still unclear. In immature (Fig. 5C) or maturing (Fig. 5D) male red seabream, on the other hand, implantation

of 11KT for one-month significantly increased the brain sbGnRH mRNA levels, while implants of E₂ or T exerted no effect. 11KT is considered to be the major androgen of male red seabream, therefore, a positive feedback of 11KT on brain sbGnRH mRNA plays an important physiological role in the annual reproductive cycle of males.

Negative feedback effects of sex steroids on the GnRH system have been reported in only one species; in territorial male African cichlids, castration enlarged cell size of POA GnRH neurons (Francis et al., 1992) and 11KT and T prevented the increase of cell size of castrated males (Soma et al., 1996). It is still under debate if the same steroid (11KT) exerts both positive and negative effects on the GnRH activity. It is likely that the status of fish, such as its gonadal maturity, determines the direction (negative or positive) of the feedback effects of sex steroids.

Neurotransmitters

Although, it is still unclear how these environmental and physiological factors influence the reproductive endocrine system, several neurotransmitters, such as monoamines, amino acids or neuropeptides contained in fish brain (see review by Yu et al., 1997) are candidates that mediate information from these factors. Several reviews are available about the regulation of GnRH activities by neurotransmitters (Peter et al., 1991; Yu et al., 1997), therefore we are going to mention only about our recent work on red seabream. As the first step to understand the central regulation of the preoptic sbGnRH neuronal system of red sea bream, we have conducted experiments concerning the effects of serotonin (5-HT), GABA and neuropeptide Y (NPY) on the *in vitro* release of sbGnRH from slices of the preoptic–anterior hypothalamus (P-AH) and pituitary (Senthilkumaran et al., 2001). 5-HT, GABA and NPY all stimulated the release of sbGnRH from the P-AH but not from the pituitary of immature red seabream. They also stimulated sbGnRH release from the P-AH with a similar potency during the course of gonadal development. Specific agonists and/or antagonists of 5-HT, GABA and NPY showed that 5-HT and GABA utilize 5-HT₂ and GABA_A receptor subtypes, respectively, to mediate their action, and that NPY employs at least NPY_{Y1} and NPY_{Y2} receptor subtypes to stimulate sbGnRH release. Furthermore, combinations of different antagonists for 5-HT, GABA and noradrenaline/adrenaline did not block the stimulatory influence of NPY on the release of sbGnRH, and the brain distribution of NPY-ir neurons overlapped with sbGnRH neurons in red seabream (Okuzawa et al., unpublished result). These data indicate that the action of NPY on the sbGnRH neuronal system is probably direct. Positive effects of 5-HT and NPY on *in vitro* GnRH release from P-AH have also been reported in goldfish (Yu et al., 1991; Peng et al., 1993).

Cloning and characterization of the GnRH receptor gene

GnRH exerts its efficacies through binding to the GnRH receptor (GnRH-R), a plasma membrane-bound receptor belonging to the family of G protein-

coupled receptors (GPC-Rs). To date, cDNAs encoding GnRH-R have been cloned from several vertebrates including some teleosts. However, still limited information is available as regards the regulatory mechanisms of the expression of GnRH-R in teleosts. Therefore, we cloned a cDNA encoding GnRH-R from the pituitary of the red seabream using a RACE method, and performed some experiments in order to clarify the regulatory mechanisms of GnRH-R, which is important not only for understanding the basic reproductive physiology of teleosts but also for artificial breeding of fishes in aquaculture.

The cloned GnRH-R of the red seabream (PmGnRH-R) cDNA contained an open reading frame of 1245 bps encoding 415 amino acid residues (Okuzawa et al., unpublished results). Hydrophobicity analysis of the deduced amino acid sequence indicated the presence of seven transmembrane-spanning domains, a characteristic of GPC-Rs. Unlike mammalian GnRH-Rs but similar to other non-mammalian GnRH-Rs, the PmGnRH-R contains an intracellular carboxy-terminal tail, indicating that signal transduction mechanisms may in part differ between piscine and mammalian GnRH-Rs. When the PmGnRH-R deduced amino acid sequence was compared to GnRH-Rs isolated from the pituitary of other animals, it has the highest identity (87%) with striped bass GnRH-R (Alok et al., 2000) followed by medaka GnRH-R1 (73%) (Okubo et al., 2001), bullfrog GnRH-R1 (57%) (Wang et al., 2001), *Xenopus* (42%) (Troskie et al., 2000), Japanese eel GnRH-R (42%) (Okubo et al., 2000b), catfish GnRH-R (41%) (Tensen et al., 1997), goldfish GfB (40%), goldfish GfA (37%) (Illing et al., 1999), mouse (34%) (Tsumi et al., 1992), and other mammalian GnRH-Rs. A phylogenetic tree, generated by the UPGMA method, revealed that PmGnRH-R was clustered with the GnRH-Rs of evolutionally advanced fishes, striped bass and medaka, and an amphibian, the bullfrog. On the other hand, GnRH-Rs of phylogenetically older species, catfish, goldfish and eel, were included in an other lineage.

Exon/intron organizations of the transcribed regions were determined by sequence analyses of the PmGnRH-R gene. The PmGnRH-R gene is composed of four exons and three introns that are located in the 5' untranslated region, transmembrane domain

IV and the third cytoplasmic loop. Interestingly, this exon/intron structure is the same as the GnRH-R gene of *Xenopus laevis* (Troskie et al., 2000), and different from medaka GnRH-R1 that possessed only two introns (Okubo et al., 2001).

We characterized PmGnRH-R pharmacologically using a mammalian cell line (293 cells) (Okuzawa et al., unpublished results). A colorimetric assay for measuring the activation of G protein-coupled signaling pathways (Chen et al., 1995) was applied for the characterization of PmGnRH-R. A PmGnRH-R construct was prepared using a mammalian expression vector (pcDNA 3.1), which possessed the CMV promoter, and co-transfected to the 293 cells with a reporter plasmid (pCRE/ β -gal), in which the bacterial β -galactosidase (β -gal) gene was under the control of a promoter containing five cyclic AMP response elements (CREs). Transfected cells were incubated for 48 hours and stimulated by several GnRHs including the three native GnRHs of red sea bream and other neuropeptides (isotocin and arginine vasotocin) for 6 hours. Then, the production of β -gal was estimated. The three native GnRHs of red seabream, sGnRH, cGnRH-II, and sbGnRH increased β -gal production in a dose dependent manner. On the other hand, isotocin and arginine vasotocin showed very weak or no stimulation. These data clearly showed that the cloned PmGnRH-R is functional and specific to GnRH. In this assay, sGnRH and cGnRH-II were about 10-fold more effective than sbGnRH, a putative endogenous ligand. Similarly, cGnRH-II was more effective than endogenous preoptic GnRH in a similar pharmacological experiments in *Xenopus* (Troskie et al., 2000), catfish (Tensen et al., 1997), goldfish (Illing et al., 1999), African cichlid (Robison et al., 2001), and medaka (Okubo et al., 2001).

We studied the expression of the PmGnRH-R mRNA in several tissues of red seabream including pituitary, brain, ovary, testis, liver, kidney, heart, stomach, and muscle using RNase protection assay, and found that PmGnRH-R mRNA was expressed only in the pituitary. Hence, it is likely that the endogenous ligand of PmGnRH-R is sbGnRH, a hypophysiotropic form of GnRH among the three native GnRHs in red seabream, which is dominantly contained in the pituitary. However, it cannot be ruled out that small amounts of mRNA of GnRH-R,

below the detection limit of the RNase protection assay, were expressed in extrahypophyseal tissues as reported in other teleosts (Illing et al., 1999; Alok et al., 2000; Madigou et al., 2000; Okubo et al., 2000b; Robison et al., 2001).

Seasonal fluctuation of the expression of red sea bream GnRH receptor

As a basis for understanding the regulatory mechanism of GnRH-R expression, we determined the seasonal fluctuation of the PmGnRH-R mRNA levels in the pituitary of red seabream by a quantitative PCR method (Mori et al., unpublished results). PmGnRH-R mRNA levels were maintained at high levels during winter and early spring and reached a peak one (females) or two months earlier (males) than the onset of the spawning season, then the levels suddenly decreased from March to April (spawning season) and reached the lowest levels. After the spawning season, the levels gradually increased from May to August. It is an interesting finding that the expression of mRNA of GnRH-R is the lowest during the spawning season. A negative feedback mechanism may take part in this phenomenon. We will discuss this issue in the next section.

Regulation of the GnRH receptor

How is the mRNA expression of GnRH-R in red seabream controlled? To answer this question, we have conducted experiments about the effect of three possible environmental or physiological factors, namely water temperature, GnRH and sex steroids.

GnRH

Immature juvenile red seabream of one-year-old were implanted intramuscularly with a cholesterol pellet containing GnRH agonist (200 μ g/kg body weight). Control fish received a cholesterol pellet without the GnRH agonist. Ten days or 20 days later, the fish were killed and the PmGnRH-R mRNA levels in the pituitaries were estimated using the RNase protection assay (Kumakura et al., unpublished results). The GnRH agonist treated groups showed significantly higher GnRH-R mRNA levels

compared to the control groups. This result suggests that GnRH itself is a positive regulator of its own receptor. A study of GnRH-R binding assay in goldfish support this notion; *in vivo* injection of GnRH agonist increased binding capacity of GnRH-R in the pituitary (Omeljaniuk et al., 1989). However, the involvement of gonadal factors, such as steroids or activin is also possible in this positive effect, because the experiments were conducted *in vivo*. Further *in vitro* and/or gonadectomy studies are required to determine if the positive effect of GnRH on GnRH-R expression is direct or indirect.

Sex steroids and water temperature

In December, one-year-old juvenile fish of mixed sex were implanted intraperitoneally with silastic capsules containing E₂, 11KT or solvent. Then, the rearing temperature was increased from 15°C to 20°C, and fish were reared for one month. At the end of the experiment, the mRNA levels of PmGnRH-R in the pituitary were estimated by RNase protection assay (Kumakura et al., unpublished results). The PmGnRH-R mRNA levels of the control group which were implanted with silastic capsule containing solvent was significantly higher than the initial control fish. This suggests that the elevation of water temperature increases the transcription of the PmGnRH-R gene. On the other hand, the levels of PmGnRH-R mRNA of sex steroids (E₂ or 11KT) treated fish were significantly lower than the control fish. This suggests that steroids exert a negative effect on the GnRH-R mRNA expression. Similar positive and negative effects were reported in the three-spined stickleback, *Gasterosteus aculeatus*, and African catfish, respectively, using GnRH-R binding assay. In the three-spined stickleback, an exposure to warm temperature (20°C) in winter increased the binding capacity of GnRH-R in the pituitary irrespective of the photoperiod (Andersson et al., 1992). In African catfish, castration in males resulted in a two-fold increase in the pituitary GnRH receptor binding capacity, and replacement with androstendion reversed the increase of the binding capacity (Habibi et al., 1989).

Besides sex steroids, insulin-like growth factor I (IGF-I) or leptin are other candidates as regulators of GnRH-R, because IGF-I (Huang et al., 1998; Weil et

al., 1999) and leptin (Peyon et al., 2001) affect GTH release *in vitro* in teleosts.

Possible model for the regulatory mechanism of the onset of puberty and seasonal reproductive cycle of red seabream

Onset of puberty

Normally, red seabream spawns when they become two-year-old (2+), and underyearling (0+) or one-year-old (1+) red seabream do not spawn even if they are subjected to suitable environmental conditions in the spawning season. In other words, prepubertal 0+ or 1+ fish do not respond to suitable environmental cues for gonadal maturation. Implant of GnRH agonist into 1+ fish, however, induces gonadal maturation and ovulation (Kumakura et al., unpublished results). Therefore, the pituitary–gonad axis is already functional in 1+ fish, while the GnRH neuronal system and/or higher central nervous system (CNS) controlling reproduction is not developed or functional yet in 0+ and 1+ red seabream. Why the reproductive brain, namely the GnRH system and higher CNS, of 0+ to 1+ prepubertal fish does not respond to environmental cues? There are two possibilities; (1) the system of the reproductive brain is not completely developed. (2) an unknown inhibitor mechanism exists in prepubertal fish. Morphologically, the sbGnRH neuronal system of juvenile fish of 37 days after hatching is similar to adult fish, therefore it is likely that the sbGnRH neuronal system of 1+ fish is completed. However, we do not know about the higher CNS controlling sbGnRH system. Also, no evidence is available about any inhibitory factor. If an inhibitor mechanism exists under the regulation of puberty, somatic growth may be a required condition in order to overcome this inhibition. The mediators of somatic growth, such as IGF-I or leptin, are candidates that could feedback centrally and inactivate the inhibitor mechanism.

Seasonal reproductive cycle

After two years from hatching, red seabream reaches the size capable of puberty; 2+ fish can respond to suitable environmental conditions. In late autumn, a decrease of water temperature to the optimum in-

duces gonadal recrudescence through activating the CNS–GnRH system. Increase in photoperiod and/or water temperature from winter to spring enhances gonadal maturation. The increase of sbGnRH secretion underlies the gonadal maturation in this season; stimuli of long photoperiod and/or optimum temperature are accepted by sensory organs, and activate the CNS regulating sbGnRH secretion. In turn, secreted sbGnRH not only stimulates GTH release but also increases the expression of its own receptor, therefore GTH secretion is largely enhanced. In addition, the increase in water temperature in early spring also increases the GnRH-R expression. In accordance with the increase in GTH levels, production of sex steroids is activated and the steroids feedback centrally and up-regulate sbGnRH synthesis. On the other hand, steroids negatively feedback to GnRH-R synthesis and maintain GTH levels at adequate concentrations. Further increase in the water temperature in early summer inactivates sbGnRH synthesis and/or release and in turn reduced the activity of the entire brain–pituitary–gonad axis, and terminates the spawning season in red seabream.

Still a lot of speculation is contained in this model, thus the following studies are required in order to validate this model; (1) to test if environmental factors do affect sbGnRH synthesis and release, (2) to clarify the CNS mediating the effect of environmental factors and internal factors such as sex steroids, (3) to determine whether the effect of water temperature on GnRH-R expression is direct or indirect, namely mediated via sbGnRH secretion.

Acknowledgements

The research from our laboratory described in this chapter was supported in part by a Bio-Design Program (BDP-02-IV-2-3) from the Ministry of Agriculture, Forestry and Fisheries of Japan.

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CHAPTER 9

Multiplicity of gonadotropin-releasing hormone signaling: a comparative perspective

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Introduction

Gonadotropin-releasing hormone (GnRH) is best known for its regulation of the synthesis and secretion of pituitary gonadotropin hormones (GtH), which include follicle-stimulating hormone (FSH) and luteinizing hormone (LH). The production of LH and FSH is also regulated by other neuromodulatory factors including, steroids, catecholamines, amino acids and gonadal peptides (Chang and Jobin, 1994; Shupnik, 1996; Van Der Kraak et al., 1998). In addition to its effects on the pituitary, GnRH exerts effects in other peripheral tissues including the brain, gonads and placenta (King and Millar, 1995). The signal transduction of GnRH-induced GtH synthesis and secretion has been studied extensively in mammals (Naor, 1990; Stojilkovic et al., 1994; Stojilkovic and Catt, 1995; Naor et al., 1998; Shacham et al., 1999) and in non-mammalian vertebrates, in particular, GnRH-induced control of GtH release in fishes (Chang and Jobin, 1994; Chang et al., 1996b, 2000). The objective of this review is to summarize the information on the mechanisms of GnRH-induced GtH release and subunit gene expression

from a comparative perspective with emphasis on the studies carried out on goldfish (Tables 1 and 2). We will also examine the evidence for the functional significance of GnRH and GnRH receptor multiplicity in vertebrates.

Multiplicity of GnRH and its receptor

The primary sequences of 14 molecular forms of GnRH have been characterized in various vertebrate and protochordate species (Montaner et al., 2001). All vertebrate classes were found to have two or more forms of GnRH, including one or two molecular forms of GnRH as well as chicken (c)GnRH-II which appears to be ubiquitous in the vertebrate classes studied (King and Millar, 1995; Millar et al., 1997). There is evidence for differential distribution and functional diversity of GnRH variants in the brain–pituitary axis, as well as, neural and peripheral tissues in vertebrates (King and Millar, 1995; Pati and Habibi, 1998; Habibi and Matsoukas, 1999). For example, goldfish brain and pituitary contains salmon (s)GnRH and cGnRH-II (Yu et al., 1988). Both GnRH forms act at the level of the pituitary to stimulate the synthesis and release of GtHs in the goldfish (Chang et al., 1990; Khakoo et al., 1994; Klausen et al., 2001). The discovery of cGnRH-II in the brains of mammals (including humans), and a cGnRH-II specific receptor in the pituitary suggest that cGnRH-II may also have hypophysiotrophic function in mammals (Lescheid et al., 1997; Chen

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TABLE 1

Signal transduction of GnRH-induced FSH and LH synthesis and secretion^a

	Release				Gene expression				
	PKC	Ca ²⁺	AA	PKA	PKC	Ca ²⁺	AA	ERK	PKA
FSH									
Rat pit. cells	Y ¹ N ⁵ Y ⁶	Y ² — —	Y ² — —	— — —	Y ³ — —	N ³ — —	— — —	Y ⁴ — —	— — —
Rat hemipit.	Y ⁶	—	—	—	—	—	—	—	—
Rat-Luc (GGH ₃)	—	—	—	—	Y ⁷	N ⁷	—	—	—
Ovine pit. cells	—	Y ⁸	—	—	—	—	—	—	—
Ovine-Luc (HeLa)	—	—	—	—	Y ⁹	—	—	—	—
Frog hemipit.	—	Y ¹⁰	—	—	—	—	—	—	—
Goldfish pit. cells	—	—	—	—	—	—	—	—	—
sGnRH	—	—	—	—	N ¹¹	—	—	—	—
cGnRH-II	—	—	—	—	N ¹¹	—	—	—	—
Tilapia pit. cells	—	—	—	—	N ¹²	—	—	N ¹³	Y ¹²
LH									
Rat pit. cells	Y ^{5,14} N ¹⁷ Y ⁶	Y ^{2,15} — —	Y ² — —	— — —	Y ³ Y ¹⁷ —	Y ^{3,16} — —	— — —	N ⁴ — —	— — —
Rat hemipit.	Y ⁶	—	—	—	—	—	—	—	—
Rat-Luc (GGH ₃)	—	—	—	—	Y ⁷	Y ⁷	—	—	—
Rat-Luc (αT3-1)	—	—	—	—	—	Y ¹⁶	—	N ¹⁶	—
Equine-Luc (αT3-1)	—	—	—	—	Y ¹⁸	N ¹⁸	—	Y ¹⁸	—
Ovine pit. cells	—	Y ⁸	—	—	—	—	—	—	—
Chicken pit. cells	Y ¹⁹	Y ^{20,21}	—	—	—	—	—	—	—
Frog hemipit.	—	Y ¹⁰	—	—	—	—	—	—	—
Goldfish pit. cells	—	—	—	—	—	—	—	—	—
sGnRH	Y ²²	Y ²²	Y ²²	N ²²	N ¹¹	—	—	—	—
cGnRH-II	Y ²²	Y ²²	N ²²	N ²²	N ¹¹	—	—	—	—
Tilapia pit. cells	Y ²³	—	—	Y ²³	Y ²³	—	—	Y ¹³	Y ²³
Tilapia pit. fragments	—	Y ²⁴	—	—	—	—	—	—	—
Murrel pit. cells	—	Y ²⁵	—	—	—	—	—	—	—

^a Superscript numbers refer to references and (—) denotes unknown or not determined. ¹ Wiebe et al., 1994; ² Chang et al., 1988; ³ Ben-Menahem and Naor, 1994; ⁴ Haisenleder et al., 1998; ⁵ Audy et al., 1990; ⁶ Johnson et al., 1992; ⁷ Saunders et al., 1998; ⁸ Kile and Nett, 1994; ⁹ Strahl et al., 1998; ¹⁰ Porter and Licht, 1986; ¹¹ C. Klausen, J.P. Chang, H.R. Habibi, unpublished; ¹² Yaron et al., 2001; ¹³ Gur et al., 2001a; ¹⁴ Stojilkovic et al., 1988b; ¹⁵ Stojilkovic et al., 1988a; ¹⁶ Weck et al., 1998; ¹⁷ Andrews et al., 1988; ¹⁸ Call and Wolfe, 1999; ¹⁹ Johnson and Tilly, 1991; ²⁰ Smith et al., 1987; ²¹ Davidson et al., 1988; ²² Chang et al., 1996b; ²³ Melamed et al., 1996; ²⁴ Levavi-Sivan and Yaron, 1989; ²⁵ Jamaluddin et al., 1989.

et al., 1998; Millar et al., 2001; Neill et al., 2001) similar to that described previously for goldfish (Yu et al., 1988; Lin and Peter, 1996; Peter and Yu, 1997). Thus, understanding the functional aspects of multiple GnRH forms in a single target tissue is of increasing interest and urgency. To date, information on functional aspects of multiple forms of GnRH in a single target tissue has been largely derived from non-mammalian models, and especially from studies in teleost fishes. Among these, studies on the goldfish in particular have generated novel ideas on the functional significance of multiple GnRH forms.

The existence of multiple forms of GnRH represents more than 500 million years of evolution and apparently involves parallel evolution of GnRH receptors. To date, the existence of three types of GnRH receptors (Type I, Type II and Type III) with different structures and ligand selectivity have been demonstrated in the pituitary and extrapituitary tissues in a number of vertebrates. In mammals, including human, there is evidence for the existence of two GnRH receptor subtypes, Type I and Type II, with different structure and ligand selectivity (Millar et al., 2001; Neill et al., 2001). The type II GnRH

TABLE 2

Signal transduction of GnRH-induced GtH- α and GH synthesis and secretion^a

	Release				Gene expression				
	PKC	Ca ²⁺	AA	PKA	PKC	Ca ²⁺	AA	ERK	PKA
GtH- α									
Human-Luc (GGH ₃)					Y ¹	Y ¹	—	—	—
Human-Luc (α T3-1)					Y ²	Y ^{3,4}	—	Y ²	—
Human-Luc (rat pit.)					—	—	—	Y ²	—
Human-Luc (HeLa)					Y ⁵	—	—	—	—
Rat pit. cells	—	—	—	—	Y ⁶	Y ⁶	—	Y ⁷	—
	—	—	—	—	—	N ⁸	—	—	—
Rat-Luc (α T3-1)					—	N ⁸	—	Y ⁸	—
Mouse (α T3-1)	—	Y ³	—	—	Y ⁹	Y ⁹	Y ¹⁰	—	—
Mouse-Luc (α T3-1)					—	—	—	Y ¹¹	—
Goldfish pit. cells									
sGnRH	—	—	—	—	N ¹²	—	—	—	—
cGnRH-II	—	—	—	—	N ¹²	—	—	—	—
Tilapia pit. cells	—	—	—	—	Y ¹³	—	—	Y ¹⁴	Y ¹³
GH									
Goldfish pit. cells									
sGnRH	Y ¹⁵	Y ¹⁵	N ¹⁵	N ¹⁵	N ¹²	—	—	—	—
cGnRH-II	Y ¹⁵	Y ¹⁵	N ¹⁵	N ¹⁵	N ¹²	—	—	—	—
Tilapia pit. cells	Y ¹⁶	—	—	N ¹⁶					

^a Superscript numbers refer to references and (—) denotes unknown or not determined. ¹ Saunders et al., 1998; ² Sundaresan et al., 1996; ³ Holdstock et al., 1996; ⁴ Call and Wolfe, 1999; ⁵ Strahl et al., 1998; ⁶ Ben-Menahem and Naor, 1994; ⁷ Haisenleder et al., 1998; ⁸ Weck et al., 1998; ⁹ Ben-Menahem et al., 1995; ¹⁰ Ben-Menahem et al., 1994; ¹¹ Roberson et al., 1995; ¹² C. Klausen, J.P. Chang, H.R. Habibi, unpublished; ¹³ Gur et al., 2001b; ¹⁴ Gur et al., 2001a; ¹⁵ Chang et al., 1996b; ¹⁶ Melamed et al., 1996.

receptor was found to be selective for cGnRH-II with somewhat different signal transduction mechanisms (Millar et al., 2001; Neill et al., 2001). Two subtypes of GnRH receptor (GfA and GfB) have been identified in the goldfish pituitary and these are expressed also in the gonads (Illing et al., 1999). Experimental evidence suggests that both GfA and GfB have similar ligand selectivity and are isoforms of the Type I GnRH receptor family (Troskie et al., 1998; Wang et al., 2001). Three pure antagonists of the human GnRH receptor acted as full or partial agonists for a recently cloned chicken pituitary GnRH receptor (Sun et al., 2001). In addition, recent findings on the physiological role of GnRH in the autocrine/paracrine control of ovarian function suggests that there may be additional forms of GnRH receptors with different ligand selectivity and coupling to signal transduction mechanisms in goldfish (Pati and Habibi, 2000, 2002; Habibi et al., 2001). Indeed, evidence is increasing in support of a functional role for GnRH and GnRH receptor plurality.

Protein kinase C (PKC)

GnRH-induced GtH release

Membrane bound phosphatidylinositol-specific phospholipase C (PLC) is highly selective for phosphatidylinositol 4,5-bisphosphate and catalyzes its cleavage yielding cytosolic inositol 1,4,5-trisphosphate (IP₃) and membrane-soluble diacylglycerol (DAG). IP₃ is involved in the release of calcium (Ca²⁺) from intracellular stores via specific IP₃ receptors. The primary role of DAG is to stimulate the activity of certain isoforms of PKC. DAG can also be generated by pathways involving phosphatidylcholine-specific PLC and phospholipase D (PLD) (Berridge, 1987; Rasmussen et al., 1995).

In rat pituitary gonadotropes, GnRH treatment results in a rapid rise in the levels of DAG, IP₃ and other inositol phosphates (IPs) (Andrews and Conn, 1986; Morgan et al., 1987; Naor, 1990; Horn et al., 1991; Stojilkovic et al., 1994). This action requires

a pertussis toxin-insensitive G-protein (G_q and/or G_{11}) which couples receptor activation to the stimulation of $PLC\beta$ (Hsieh and Martin, 1992; Grosse et al., 2000). GnRH receptors may also couple to other G-proteins including, G_i and G_s (Stanislaus et al., 1998). A delayed activation of PLD by GnRH, possibly via PKC , is also observed in $\alpha T3-1$ cells and may contribute additional DAG for the activation of PKC (Stojilkovic et al., 1994; Stojilkovic and Catt, 1995; Naor et al., 1998). Johnson et al. (1992) reported that the PKC inhibitors, staurosporine and H7, reduced GnRH-stimulated FSH or LH release from rat hemipituitaries. In another report, PKC depletion did not affect GnRH-induced LH release in primary cultures of rat pituitary cells (Andrews et al., 1988). Also in rat pituitary cells, Audy et al. (1990) found that PKC depletion reduced GnRH-stimulated LH but not FSH release. While there are contradictory reports regarding the involvement of PKC in GnRH-induced GtH release, there is sufficient evidence for the involvement of PKC in the GnRH-induced response in rat and in a number of other vertebrates. GnRH stimulates the redistribution of PKC and the effects of GnRH on FSH and LH release can be reduced by inhibition and depletion of PKC (Chang et al., 1987; Stojilkovic et al., 1988b; Naor, 1990; Wiebe et al., 1994). PKC depletion effectively reduced chicken GnRH-I-induced LH secretion in primary cultures of dispersed chicken pituitary cells (Johnson and Tilly, 1991). In goldfish, the use of PKC inhibitors and cells depleted of PKC has provided sound evidence for the involvement of PKC in acute and prolonged sGnRH- and cGnRH-II-stimulated GtH release (Chang et al., 1991a; Jobin et al., 1993). Similarly, PKC is also involved in sGnRH-induced GtH secretion in primary cultures of dispersed tilapia pituitary cells (Melamed et al., 1996). Interestingly, the cGnRH-II-stimulated GtH response in goldfish was more sensitive to inhibition by a PKC inhibitor than that to sGnRH (Chang et al., 1991a). In this context, there is increasing evidence for the existence of different PKC -sensitive pathways mediating the action of GnRH variants. Some of these possibilities are discussed in the following section on PKC multiplicity and signaling diversity.

GnRH-induced GtH gene expression

Available data suggest that PKC also mediates GnRH effects at the level of GtH gene expression. In cultured rat pituitary cells, PKC depletion and treatment with GF109203X (PKC inhibitor) inhibited the stimulation of GtH- α , FSH- β and LH- β mRNA levels by GnRH (Andrews et al., 1988; Ben-Menahem and Naor, 1994). Selective deletions of the GtH- α promoter linked to a luciferase reporter gene in transfected $\alpha T3-1$ cells demonstrated co-localization of responsiveness to GnRH and the phorbol ester (PMA) (Schoderbek et al., 1993). Endogenous GtH- α mRNA levels were found to be increased following treatment with GnRH and phorbol esters in $\alpha T3-1$ cells. The effect of GnRH could be inhibited by GF109203X and by phorbol ester-induced depletion of PKC . The responses to GnRH and PMA or the Ca^{2+} ionophore, ionomycin, were not additive; neither were the responses to PMA and ionomycin. These data indicate that PKC and Ca^{2+} probably act sequentially or converge in the regulation of GnRH-induced GtH- α gene expression. This idea is further reinforced by the observation that PKC depletion and incubation in Ca^{2+} -free medium abolished the responses to ionomycin and PMA, respectively (Horn et al., 1991; Ben-Menahem et al., 1995). PKC depletion reduced GnRH-induced human GtH- α -luciferase activity in $\alpha T3-1$ cells (Sundaresan et al., 1996). In GGH₃ cells (GH₃ cells stably expressing the GnRH receptor) transfected with luciferase constructs containing promoters for human GtH- α , rat FSH- β and rat LH- β , GF109203X reduced GnRH-induced luciferase activity for all three constructs (Saunders et al., 1998). In $\alpha T3-1$ cells, GnRH-stimulated expression of a luciferase construct containing the equine LH- β promoter was reduced by PKC depletion and GF109203X (Call and Wolfe, 1999). Similarly, GF109203X reduced GnRH-induced expression of an ovine FSH- β -luciferase or a human GtH- α -luciferase construct in HeLa cells transfected with the mouse GnRH receptor (Strahl et al., 1998). The mechanism by which GnRH-induced PKC activity regulates gene expression is not well understood. Recent studies using phorbol esters have demonstrated that the early growth response gene 1 product may be involved in mediating PKC -induced effects of GnRH on LH- β gene expres-

sion (Halvorson et al., 1999; Tremblay and Drouin, 1999). Currently, the investigation of the regulatory elements contained within the promoter regions of GtH subunit genes is an area of intense study.

There is also a limited amount of information in non-mammalian vertebrates. In dispersed tilapia pituitary cells, GF109203X effectively reduced sGnRH-induced GtH- α and LH- β , but not FSH- β mRNA levels (Melamed et al., 1996; Gur et al., 2001b; Yaron et al., 2001). In goldfish, PKC is involved in the negative regulation of basal GtH subunit gene expression and appears not to be involved in GnRH-induced increases in GtH- α , FSH- β or LH- β mRNA levels (Klausen et al., 2000; C. Klausen, J.P. Chang, H.R. Habibi, unpublished).

When the results discussed above are viewed together, it is clear that the involvement of PKC in the regulation of LH release by GnRH has been confirmed in a variety of cell types and species. However, the role of PKC in GnRH-induced FSH release is not as well understood, in part, due to the lack of a specific antibody for FSH. The involvement of PKC in GnRH-induced GtH subunit gene expression appears to be common in a number of species. This is not surprising since GnRH is coupled to the PLC-activating $G_{q/11}$ G-proteins. In addition, the dual (PKC/ Ca^{2+}) nature of PLC-mediated signaling provides GnRH with capacity to regulate many physiological processes. In tilapia, the lack of PKC involvement in sGnRH-induced FSH- β gene expression may reflect differences in the 5' regulatory regions of the various GtH genes in this species. However, the possibility that GnRH or GnRH receptor multiplicity may be responsible has not been investigated. As discussed later, PKC also plays a central role in the activation of mitogen-activated protein kinases by GnRH in gonadotropes.

PKC multiplicity and signaling diversity

Structural properties and co-factor requirements have led to the classification of PKCs into three groups. Conventional PKCs include the α , βI , βII and γ isoforms which are activated by Ca^{2+} , DAG and phosphatidylserine. Novel PKC isoforms (δ , ϵ , η and θ) lack the characteristic Ca^{2+} -binding domain and are activated by DAG, phosphatidylserine and unsaturated fatty acids. The ζ and $\lambda(\iota)$ isoforms

comprise the third group referred to as the atypical PKCs. They may have some constitutive activity but are insensitive to Ca^{2+} or DAG and are activated by phosphatidylserine, phosphatidylinositides or unsaturated fatty acids (Liu and Heckman, 1998). In mammalian cells, GnRH-induced activation of PLC is followed closely by the activation of PLD and phospholipase A_2 (PLA $_2$). It has been hypothesized that DAG and Ca^{2+} , derived from the initial activation of PLC, may be responsible for the rapid activation of conventional PKCs involved in secretion. The subsequent activation of PLD and PLA $_2$ would generate signaling molecules for the activation of novel PKCs or atypical PKCs that could be implicated in prolonged release and gene expression (Naor et al., 1998). As such, the diversity in GnRH signaling may be the result of differential activation of PKC isoforms. The report that inhibition of DAG lipase and subsequent increase in DAG levels did not elevate LH and FSH secretion supports this hypothesis (Chang et al., 1988). It indicates that a specific PKC isoform requiring simultaneous signals from both DAG and Ca^{2+} or some other signaling molecules may be involved in the regulation of hormone secretion. While pituitary cells have been shown to express PKC α , βII , δ , ϵ and ζ , little is known about the roles of specific PKC isoforms in GnRH-induced GtH release or synthesis (Naor et al., 1998; Shacham et al., 1999). Isoform specificity has been demonstrated in digitonin permeabilized rat pituitary cells where the addition of α or β , but not γ PKC, was able to recover PMA-induced GtH secretion (Naor et al., 1989). Together these results provide evidence for the role of distinct PKC isoforms in the functional specificity of GnRH signaling. The differential recruitment of PKCs may provide the cell with the ability to differentiate between stimuli that may or may not require the up-regulation of gene expression. In this context, certain isoforms or sets of isoforms may be responsible for a diverse array of GnRH-mediated events. It is possible that distinct GnRH receptor subtypes with differential ligand selectivity, generating dissimilar second messengers may independently regulate cellular processes by activating different PKC isoforms. Although largely untested, there is evidence for GnRH receptor subtypes with differential signaling in goldfish gonadotropes (discussed below).

The role of calcium

GnRH-induced GtH release

In mammals, treatment of pituitary cells with GnRH results in the mobilization of Ca^{2+} from IP_3 -sensitive stores followed by the influx of extracellular Ca^{2+} via L-type voltage-sensitive Ca^{2+} channels (VSCCs). Indeed, Ca^{2+} mobilization from IP_3 -sensitive stores was found to be necessary for initial rapid phase of GtH secretion whereas sustained GnRH-induced LH release relies in part on the influx of Ca^{2+} from the extracellular environment via VSCCs (Naor, 1990; Naor et al., 1998). In rat pituitary cells, the stimulation of FSH and LH release by GnRH is reduced in Ca^{2+} -free medium and in the presence of VSCC blockers (Chang et al., 1986; Chang et al., 1988; Stojilkovic et al., 1988a; Blotner et al., 1990). In $\alpha\text{T3-1}$ cells, thapsigargin but not nifedipine was able to reduce GnRH-stimulated secretion of GtH- α subunit indicating a role for intracellular but not extracellular Ca^{2+} (Holdstock et al., 1996). Incubation of ovine pituitary cells in Ca^{2+} -free medium abolished the secretion of LH and FSH in response to GnRH (Kile and Nett, 1994). VSCC blockers and Ca^{2+} -free medium have been used to demonstrate the involvement of Ca^{2+} in GnRH-stimulated LH release from dispersed chicken pituitary cells (Smith et al., 1987; Davidson et al., 1988). Incubation of frog hemipituitaries in Ca^{2+} -free medium or in the presence of CoCl_2 abolished GnRH-stimulated FSH and LH secretion (Porter and Licht, 1986). In goldfish, the involvement of L-type VSCCs in acute and prolonged sGnRH- and cGnRH-II-induced LH release from primary pituitary cells has also been demonstrated pharmacologically (Chang et al., 2000). Interestingly, sGnRH and cGnRH-II differ in their abilities to generate IP_3 and their requirements for extracellular Ca^{2+} influx and intracellular Ca^{2+} stores are distinct. sGnRH and cGnRH-II also generate Ca^{2+} signals that differ in their temporal features in identified goldfish gonadotropes (Johnson et al., 1999). Treatment of [^3H]inositol-prelabeled goldfish pituitary cells with sGnRH was found to increase levels of IP_1 , IP_2 , IP_3 and other higher IP s while cGnRH-II only elevated levels of IP_2 . The lack of IP_3 production by cGnRH-II agrees with evidence indicating that the effect of cGnRH-II on LH re-

lease is more dependent on influx of extracellular Ca^{2+} than that of sGnRH (Jobin and Chang, 1992; Chang et al., 1995). These data are also consistent with the findings that, unlike the response to sGnRH, cGnRH-II stimulation of LH release is independent of mobilization of Ca^{2+} from Xestospongin C-sensitive IP_3 receptor channels (Jobin and Chang, 1992; Chang et al., 1995; Johnson et al., 2000). On the other hand, the LH release response to both GnRH forms requires ryanodine-sensitive Ca^{2+} stores (Johnson et al., 2000). Surprisingly, sGnRH-induced, but not cGnRH-II-elicited, LH release was abolished by prior exposure to caffeine (a common ryanodine receptor agonist) and caffeine-stimulated LH secretion was mediated by mobilization of Ca^{2+} from ryanodine- and dantrolene-insensitive stores (Johnson et al., 2000, 2002b). These data indicate that pharmacologically distinct IP_3 /Xestospongin C-, caffeine- and ryanodine-sensitive intracellular Ca^{2+} stores participate differentially in sGnRH and cGnRH-II stimulation of gonadotropes. These differences may underlie known potency and efficacy characteristics of these two GnRHs in eliciting LH secretion (Chang et al., 1990). In addition, differences in Ca^{2+} signaling may provide the substrate upon which reported seasonal differences in GnRH-stimulated LH synthesis/release and direct gonadal steroid action on these responses may be manifested (Habibi and Huggard, 1998; Lo and Chang, 1998b; Huggard-Nelson et al., 2002). Consistent with this hypothesis, the relative dependence on ryanodine-sensitive signaling in sGnRH-induced LH release varies according to the reproductive status (Johnson and Chang, 2002) and that testosterone positive-feedback action targets PKC-dependent signaling pathways leading to GnRH-stimulated LH release (Lo and Chang, 1998a).

The participation of extracellular Ca^{2+} influx and intracellular Ca^{2+} mobilization has similarly been demonstrated in several other teleosts. GnRH analog-induced secretion of GtH from tilapia pituitary fragments was found to be dependent on extracellular Ca^{2+} (Levavi-Sivan and Yaron, 1989). Similarly, in the murrelet (*Channa punctatus*), stimulation of GtH release by mammalian GnRH was found to be dependent on extracellular Ca^{2+} (Jamaluddin et al., 1989). In the African catfish, treatment with catfish GnRH or cGnRH-II increased IP s and intracellular

Ca^{2+} levels (Rebers et al., 2000). However, in these teleost species, it is not known if different GnRH forms elicit different Ca^{2+} responses and the roles of these responses in GnRH-induced LH release requires further investigation.

GnRH-induced GtH gene expression

In addition to its role in hormone secretion, Ca^{2+} is also involved in mediating GnRH-induced increases in GtH subunit mRNA levels. In cultured rat pituitary cells, ionomycin was able to mimic the GnRH-induced changes in LH- β mRNA levels, although GtH- α mRNA level was not affected after 24 hours of incubation (Ben-Menahem and Naor, 1994). In this experiment, FSH- β mRNA level was decreased up to 12 hours after treatment with a subsequent increase occurring at 24 hours. Moreover, incubation of the cells in Ca^{2+} -free medium abolished the response of GtH- α and LH- β , but not FSH- β , to GnRH. The combined effects of phorbol ester and ionomycin were distinct for the different GtH subunits (Ben-Menahem and Naor, 1994). Treatment with the VSCC blocker, nimodipine, was found to reduce GnRH-stimulated LH- β without affecting GtH- α mRNA levels and luciferase activity in cultured rat pituitary cells and transfected $\alpha\text{T3-1}$ cells, respectively (Weck et al., 1998). Ca^{2+} mobilization and influx were both demonstrated to be involved in the regulation of GtH- α mRNA levels by GnRH in $\alpha\text{T3-1}$ cells in experiments using ionomycin, Ca^{2+} -free medium and the cell-permeant Ca^{2+} chelator BAPTA/AM (Ben-Menahem et al., 1995). In $\alpha\text{T3-1}$ cells transfected with a human GtH- α -luciferase construct, treatment with VSCC blockers and incubation in Ca^{2+} -free medium reduced GnRH-induced GtH- α promoter activation; in contrast, thapsigargin selectively affected GnRH-induced GtH- α release but not the increase in promoter activity (Holdstock et al., 1996; Call and Wolfe, 1999). In $\alpha\text{T3-1}$ cells stably expressing an equine LH- β -luciferase construct, GnRH-induced expression was not dependent on extracellular Ca^{2+} (Call and Wolfe, 1999). Saunders et al. (1998) demonstrated that the VSCC agonist, BayK8644, increased only GtH- α -luciferase activity and nimodipine reduced GnRH-induced human GtH- α - and rat LH- β -, but not rat FSH- β -luciferase activity in GGH₃ cells.

The evidence presented above clearly indicates that Ca^{2+} derived from extracellular and intracellular sources may differentially mediate GnRH-induced GtH- α gene expression and secretion, respectively (Holdstock et al., 1996). On the other hand, more research will be needed to investigate the role of different sources of Ca^{2+} in GnRH-induced GtH gene expression in order to clarify the differences observed. It is possible that cell type specific differences in promoter elements may explain some of the observed variations regarding the involvement of Ca^{2+} in GtH subunit gene expression. In this context, vertebrate GtH subunit genes contain 5' regulatory regions with different complements of transcription factor binding sites (reviewed in Ando et al., 2001). It is becoming increasingly evident that the multiplicity of Ca^{2+} stores provides an intricate system of spatially and temporally distinct Ca^{2+} signals important for the differential regulation of diverse physiological processes (Berridge et al., 2000; Johnson and Chang, 2000). As such, the paucity of data regarding the relative roles of various Ca^{2+} stores in the regulation of GtH subunit gene expression needs to be addressed. In goldfish pituitary cells, intracellular and extracellular Ca^{2+} stores appear to be differentially involved in the regulation of basal LH- β subunit gene expression (J.D. Johnson, C. Klausen, H.R. Habibi and J.P. Chang, unpublished).

Arachidonic acid metabolism

GnRH-induced GtH release and gene expression

Arachidonic acid (AA) can be derived directly from phospholipids by the action of PLA₂ and indirectly through the action of phospholipases A₁, C and D in concert with a host of secondary and tertiary enzymes. To date, the rat and goldfish models are the only models in which the role of AA in GnRH-stimulated GtH release has been rigorously examined. The liberation of AA via the activation of PLA₂ by GnRH occurs in $\alpha\text{T3-1}$ cells (Naor et al., 1998). GnRH-induced LH and FSH secretion involves AA liberated through the action of PLA₂ and DAG lipase (Chang et al., 1988). Furthermore, treatment with AA was able to mimic the initial phase of the secretory response to GnRH (Chang et al., 1987). In rat pituitary cells, GnRH stimulates the metabo-

lism of AA to form leukotrienes LTC₄, LTD₄ and LTE₄ as well as 5- and 15-hydroxyeicosatetraenoic acids (Dan-Cohen et al., 1992). Moreover, lipoxygenase inhibitors and a peptidoleukotriene receptor antagonist were able to reduce GnRH-induced LH release suggesting the involvement of AA metabolites in GnRH action (Chang et al., 1987; Dan-Cohen et al., 1992).

A major difference in the involvement of AA metabolism in GnRH-induced LH release has been identified in goldfish. Mixed action and specific lipoxygenase but not cyclooxygenase inhibitors reduced sGnRH- and abolished AA-induced LH release. In contrast, none of these inhibitors reduced the response to cGnRH-II indicating that sGnRH but not cGnRH-II, in part through activation of lipoxygenase metabolites, stimulates acute and prolonged LH release (Chang et al., 1989, 1991b, 1995). The enzyme responsible for generating the AA involved in GnRH-stimulated GtH release is PLA₂. PLA₂ inhibitors reduced the sGnRH-stimulated response whereas, the DAG lipase inhibitor, U-57908, was ineffective (Chang et al., 1994a).

AA and its lipoxygenase metabolites have been implicated in the regulation of GtH subunit mRNA levels. The effect of GnRH was shown to be mimicked by AA and the 5-lipoxygenase products 5-hydroxyeicosatetraenoic acid and LTC₄ in α T3-1 cells (Ben-Menahem et al., 1994). Treatments with inhibitors of PLA₂ and 5-lipoxygenase reduced GnRH-induced elevations in GtH- α mRNA levels in α T3-1 cells, although a cyclooxygenase inhibitor was without effect (Ben-Menahem et al., 1994). In cultured rat pituitary cells, AA and LTC₄ stimulated GtH- α , FSH- β and LH- β mRNA levels; whereas, 5-hydroxyeicosatetraenoic acid stimulated only FSH- β (Ben-Menahem et al., 1994).

So far, the role of AA and its metabolites in GnRH-induced GtH release and gene expression has only been examined in the rats among mammals. In goldfish, however, major differences were observed in the involvement of AA in the sGnRH- and cGnRH-II-induced responses (Chang et al., 1996b). These observations are now very relevant to the understanding of the control of GtH synthesis and release in mammals in view of the recent identification of a GnRH receptor specific for cGnRH-II in the pituitary. The role of AA in the regulation of GtH sub-

unit gene expression by GnRH is poorly understood. The observation that 5-hydroxyeicosatetraenoic acid increases GtH- α mRNA levels in α T3-1 cells but not in cultured pituitary cells indicates that a difference may exist between the clonal and cultured pituitary cell systems. Moreover, the ability of 5-hydroxyeicosatetraenoic acid to increase only FSH- β mRNA levels suggests the existence of different signaling pathways coupling GnRH receptors to GtH release and subunit gene expression. In this context, AA represents a rather poorly understood pathway with potential functional implications in the multiplicity of GnRH signaling.

Mitogen-activated protein kinases (MAPKs)

MAPK pathways can be subdivided into three sub-families; the c-jun amino-terminal kinase/stress-activated protein kinase (JNK/SAPK) pathway, the p38 MAPK pathway and the extracellular signal-regulated kinase (ERK) pathway. MAPK pathways consist of three sequentially activated protein kinases. A serine/threonine MAPK kinase kinase phosphorylates and activates a dual specificity threonine/tyrosine MAPK kinase which in turn phosphorylates and activates a serine/threonine MAPK. Active MAPK will then phosphorylate transcription factors or further regulatory kinases. In the ERK pathway the central MAPK kinase kinase is Raf-1, the MAPK kinase is MEK and the MAPK is ERK (Lewis et al., 1998; Kolch, 2000; Pearson et al., 2001). Evidence to date indicates that MAPK signal transduction pathways are activated in response to GnRH in both primary pituitary and α T3-1 cells. Indeed, many G-protein-coupled receptors are able to activate MAPKs through a wide array of mechanisms (Van Biesen et al., 1996; Lopez-Ilasaca, 1998; Naor et al., 2000; Pierce et al., 2001).

In rats, GnRH stimulates ERK activity in vivo and in vitro (Sundaresan et al., 1996; Haisenleder et al., 1998). Treatment of α T3-1 cells with GnRH has been found to activate ERK1 and ERK2 in a MEK- and PKC-dependent manner although other Ca²⁺- and tyrosine phosphorylation-dependent components may participate in this process (Sundaresan et al., 1996; Reiss et al., 1997; Call and Wolfe, 1999; Mulvaney et al., 1999; Benard et al., 2001). PKC-dependent activation of ERKs by GnRH has

also been demonstrated in GGH₃ cells (Han and Conn, 1999). GnRH is thought to activate ERKs by two distinct pathways converging at the level of Raf-1. One pathway involves the activation of Raf-1 by PKC and another involves Src and Ras (Benard et al., 2001). The ERK pathway appears to be involved in GnRH-stimulated promoter activity of human GtH- α in primary cultures of rat pituitary cells and α T3-1 cells (Sundaresan et al., 1996). Similarly, stimulation of mouse GtH- α promoter activity by GnRH was found to involve the ERK pathway (Roberson et al., 1995). In rat pituitary cells, ERKs are involved in GnRH-induced increases in GtH- α and FSH- β but not LH- β mRNA levels (Haisenleder et al., 1998). Likewise, in α T3-1 cells transfected with rat GtH- α - or LH- β -luciferase constructs, a MEK inhibitor reduced GnRH-stimulated GtH- α but not LH- β promoter activity (Weck et al., 1998). In contrast, GnRH-induced expression of an equine LH- β -luciferase construct was found to be reduced by the MEK inhibitor PD98059 (Call and Wolfe, 1999). In addition to activating ERKs, GnRH also stimulates the activity of JNK, p38 MAPK and big MAPK (Levi et al., 1998; Roberson et al., 1999; Naor et al., 2000). Although activated by GnRH, p38 MAPK does not appear to regulate the mouse GtH- α gene expression (Roberson et al., 1999).

sGnRH was found to activate both ERK1 and ERK2 in a MEK- and PKC-dependent manner in primary cultures of dispersed tilapia pituitary cells. PD98059 inhibited sGnRH-stimulated increases in GtH- α and LH- β but not FSH- β mRNA levels (Gur et al., 2001a). The lack of PKC-dependent ERK involvement in the regulation of FSH- β mRNA levels agrees with the report that sGnRH-induced increases in FSH- β mRNA levels are unaffected by PKC inhibition (Yaron et al., 2001).

Together, these results demonstrate the differential involvement of MAPK in GnRH-induced GtH subunit gene expression between and within species. These differences may reflect variations in the elements contained within the promoter regions that govern the transcription of the different GtH subunits or other species specific differences. To date, there have been no studies examining the possibility of differential regulation by multiple GnRHs. The recently cloned mammalian Type II GnRH receptor differs from the Type I receptor in its ability to

activate p38 MAPK, although the functional significance of such a difference has not been determined (Millar et al., 2001). Currently there is no information regarding the involvement of MAPK signaling in the control of GtH release. Given that PLA₂ can be activated by ERK, a role for ERK in GnRH-induced GtH secretion could exist and needs to be investigated.

PKA and GnRH?

The production of cyclic adenosine 3',5'-monophosphate (cAMP) by adenylyl cyclase in response to receptor activation and the subsequent activation of cAMP-dependent protein kinase A (PKA) is involved in the intracellular signaling of many hormones (Beebe, 1994). In primary pituitary and α T3-1 cells, cAMP levels are not affected by GnRH treatment and as such are considered not to participate directly in GnRH-induced changes in GtH secretion or gene expression (Conn et al., 1979; Horn et al., 1991). However, there are reports of GnRH-induced cAMP production in rat hemipituitaries (Bourne, 1988), monkey COS-7 cells transfected with the mouse GnRH receptor (Arora et al., 1998) and rat GGH₃ cells (Kaiser et al., 1997). In goldfish, treatment with sGnRH or cGnRH-II did not elevate levels of cAMP, and administration of the PKA inhibitor, H89, was without effect on GnRH-induced GtH secretion (Chang et al., 1992; Jobin et al., 1996). Similarly, cAMP levels were not affected by treatment with either catfish GnRH or cGnRH-II in primary cultures of African catfish pituitary cells (Rebers et al., 2000). In tilapia, however, there is evidence for the involvement of PKA in GnRH-induced GtH synthesis and release. The PKA inhibitor H89 was found to reduce sGnRH-stimulated GtH release and subunit mRNA levels (Melamed et al., 1996; Gur et al., 2001b; Yaron et al., 2001). Although PKA is not involved directly in GnRH action in most systems, this pathway may be of importance as a modulator potentially interacting with GnRH-mediated pathways. In rat pituitary cells, forskolin, cAMP analogues and flufenamic acid have been used to demonstrate that the PKA pathway regulates GtH- α , FSH and LH secretion as well as subunit mRNA levels (Ishizaka et al., 1993; Holdstock and Burrin, 1994). In goldfish, elevation of cAMP levels or treat-

ment with forskolin can potentiate the GtH response to native GnRHs via interactions with downstream PKC- and Ca^{2+} -dependent signaling mechanisms (Chang et al., 1992; Chang et al., 2001).

GnRH and growth hormone (GH)

In goldfish, GnRH receptors are present in somatotropes and GnRH stimulates the synthesis and release of GH (Marchant et al., 1989; Cook et al., 1991; Habibi et al., 1992; Mahmoud et al., 1996; Klausen et al., 2001; C. Klausen, J.P. Chang and H.R. Habibi, unpublished). Although not universally observed (Bosma et al., 1997), GnRH binding sites and GnRH involvement in GH secretion has been reported in several other fish species (Lin et al., 1993; Melamed et al., 1996; Stefano et al., 1999), as well as in certain clinical conditions in humans (Marchant et al., 1989). GH interacts with the reproductive axis and potentiates the effects of GtH on steroidogenesis (Le Gac et al., 1993). Like LH release, PKC and extracellular Ca^{2+} are required for both acute and prolonged sGnRH- and cGnRH-II-induced GH release in goldfish (Chang and De Leeuw, 1990; Chang et al., 1991a, 1994b). In contrast, AA and its metabolites are not involved in either sGnRH- or cGnRH-II-induced GH release since inhibitors of PLA_2 , cyclooxygenase and lipoxygenase did not influence GnRH activity (Chang and De Leeuw, 1990; Chang et al., 1994b, 1996a). It should be noted that AA pathway is active in somatotropes and is involved in dopamine-induced GH release in the goldfish pituitary (Chang et al., 1996a). There are also differences in the mechanisms mediating the effects of sGnRH and cGnRH-II in the goldfish somatotropes. Only cGnRH-II action involves a ryanodine-sensitive intracellular Ca^{2+} store, although both sGnRH and cGnRH-II stimulation of GH release involve caffeine- and TMB-8-sensitive intracellular Ca^{2+} -mobilization events. Conversely, sGnRH-induced GH release, but not cGnRH-II, is dependent on Xestospongin C (IP_3)-sensitive mechanism (Johnson and Chang, 2002). Thus, it is evident that the action of both sGnRH and cGnRH-II are somehow different between somatotropes and gonadotropes, supporting the hypothesis for the existence of different GnRH receptor subtypes (or suites of receptor subtypes) in somatotropes and

gonadotropes in goldfish. In this context, amino acid substitutions in GnRH were shown to reduce its GtH-releasing potency without affecting GH-releasing activity (Habibi et al., 1992).

In tilapia, the PKC pathway is directly involved in GnRH-stimulated GH release as in the case for GtH secretion. However, unlike the GtH response, GnRH stimulation of GH secretion is unaffected by H89, a PKA inhibitor which is effective against dopamine-induced GH release (Melamed et al., 1996). Thus, the complement of GnRH receptors in gonadotropes and somatotropes appears to differ in tilapia, as in the case of the goldfish.

GnRH stimulates GH release but not mRNA levels in tilapia, demonstrating that the control of secretion and gene expression by GnRH can be completely uncoupled in this species (Melamed et al., 1996). In contrast, in goldfish, GH mRNA levels are increased following treatment with both sGnRH and cGnRH-II, *in vivo* and *in vitro* (Mahmoud et al., 1996; Klausen et al., 2001; C. Klausen, J.P. Chang and H.R. Habibi, unpublished). Currently there is no information regarding the mechanisms of GnRH-induced GH gene expression. Recent work in our labs indicates that Ca^{2+} is involved in the regulation of basal GH mRNA levels in goldfish pituitary cells. The effects of increased intracellular Ca^{2+} on GH mRNA levels were different depending on the origin of the Ca^{2+} involved, suggesting that functional specificity in GnRH signaling may involve multiplicity of Ca^{2+} mobilization (Johnson et al., 2002a).

GnRH signaling in the ovary

In addition to its hypophysiotrophic activity, GnRH is known to function as a paracrine/autocrine regulator of gonadal function. The demonstration of ovarian GnRH and GnRH receptor gene expression and their regulation by various factors in the ovary provide clear evidence that GnRH peptides are important autocrine/paracrine factors involved in the regulation of ovarian function. In mammals, GnRH directly affects follicular steroidogenesis and resumption of meiosis. While treatment with GnRH was found to stimulate basal steroidogenesis, it inhibited gonadotropin-induced cAMP and steroid production in a maturational stage-dependant manner (Hsueh and Jones, 1981; Hsueh and Schaefer, 1985; Knecht

et al., 1985; Leung and Steele, 1992). GnRH exerts direct action in the ovary through specific GnRH receptors coupled to G_q and PLC (Leung and Steele, 1992). Recent studies on isolated human granulosa cells demonstrated that GnRH actions on steroidogenesis are significantly enhanced in the presence of prostaglandin F 2α (Vaananen et al., 1997). There is a significant degree of similarity in intracellular signaling cascades mediating GnRH actions in the pituitary and ovary, including involvement of PLA $_2$, PLC and PLD as well as the activation of MAPK (Leung and Steele, 1992; Steele and Leung, 1993; Kang et al., 2001). Direct ovarian action of GnRH has also been investigated in fish and amphibians (Chieffi et al., 1991; Habibi, 1999; Habibi et al., 2001). In goldfish, GnRH exerts both stimulatory and inhibitory actions on oocyte meiosis and follicular steroidogenesis, depending upon the presence or absence of GtH. In follicle-enclosed goldfish oocytes, a number of GnRH variants including cGnRH-II, sGnRH and sGnRH analogs were found to individually stimulate oocyte meiosis in vitro as well as histone H1 kinase activity, which is an indicator of maturation promoting factor (MPF) activity (Habibi et al., 1988; Pati and Habibi, 2000; Pati et al., 2000). However, in the presence of GtH, sGnRH and a sGnRH analog were found to inhibit GtH-induced germinal vesicle breakdown, while cGnRH-II had no effect on GtH-induced responses (Habibi et al., 1988; Pati and Habibi, 2000). Interestingly, addition of a GnRH antagonist was found to effectively block the stimulatory effect of both sGnRH and cGnRH-II on oocyte meiosis, without affecting the inhibitory actions of sGnRH on GtH-induced response, suggesting the involvement of different receptors/signaling pathways mediating the stimulatory and inhibitory actions of sGnRH (Habibi and Pati, 1993; Habibi, 1999; Habibi et al., 2001). Further studies carried out in goldfish demonstrated a difference in the postreceptor mechanisms involving the stimulatory and inhibitory actions of sGnRH on reinitiation of oocyte meiosis and steroidogenesis. The stimulatory effect of both sGnRH and cGnRH-II on the reinitiation of oocyte meiosis was completely blocked by PKC inhibitors (H7 and GF109203X), suggesting the involvement of a PLC/DAG pathway in the mechanisms of GnRH-induced meiosis (Pati and Habibi, 2002). Administration of ETYA, an inhibitor

of AA metabolism only inhibited the stimulatory effect of sGnRH and had no effect on cGnRH-II-induced meiosis. Furthermore, the inhibitory effect of sGnRH on GtH-induced meiosis and steroidogenesis was completely reversed by addition of an AA metabolism inhibitor, while PKC inhibitors had no effect. There appear to be differences between the mechanisms of GnRH stimulation and the inhibition of gonadal functions in goldfish and rat (Leung and Steele, 1992). In the rat ovary, the PKC pathway and AA metabolism have been suggested to mediate the inhibitory and stimulatory effects of GnRH, respectively. Furthermore, an inhibitor of lipoxygenase, but not cyclooxygenase, was found to block GnRH and AA-induced progesterone production in the rat ovary (Wang and Leung, 1988). In contrast to the rat, cyclooxygenase metabolites have been reported to be important in the AA-induced testosterone production in the goldfish ovary (Van Der Kraak and Chang, 1990). The use of a general inhibitor of AA metabolism, ETYA reduces the products of cyclooxygenase and lipoxygenase metabolites, and could potentially increase the concentration of AA itself. Therefore, the possibility that AA might have played a direct role in reversing the inhibitory actions of sGnRH could not be ruled out.

These findings provide functional evidence in support of the hypothesis that goldfish ovarian follicles contain GnRH receptor subtypes with different ligand selectivity that mediate the stimulatory and inhibitory actions of sGnRH in the goldfish ovary. Similarly, there is evidence for the presence of different GnRH receptors, coupling independently to PLC and PLA $_2$ in the rat luteal cells (Watanabe et al., 1990).

Summary

GnRH regulation of GtH synthesis and release involves PKC- and Ca^{2+} -dependent pathways. There are differential signaling mechanisms in different cells, tissues and species. Signaling mechanisms involved in GnRH-mediated GtH release appear to be more conserved compared to that of GnRH-induced GtH gene expression. This may in part be due to different 5' regulatory regions on the GtH-subunit genes. Cell type specific expression of various signaling and/or exocytotic components may also be

responsible for the observed differences in signaling between gonadotropes and somatotropes in the goldfish and tilapia pituitaries. However, this can not explain the observed differences in post receptor mechanisms for sGnRH and cGnRH-II in gonadotropes which is more likely to result from the existence of GnRH receptor subtypes. Support for this hypothesis is also provided by observations on mechanisms of autocrine/paracrine regulation of ovarian function by sGnRH and cGnRH-II in the goldfish ovary in which GnRH antagonists only block GnRH stimulation of oocyte meiosis and do not affect inhibitory effects of sGnRH. It should be easier to explain observed variations concerning GnRH-induced responses as more information becomes available on different types of GnRH receptors, and their distribution and function in mammals and non-mammalian vertebrates.

Abbreviations

AA	arachidonic acid
BAPTA/AM	1,2-bis(<i>o</i> -aminophenoxy) ethane-N,N,N',N'-tetraacetic acid tetra (acetoxymethyl) ester
BayK8644	1,4-dihydro-2,6-dimethyl-5-nitro-4-[2'-(trifluoromethyl)phenyl]-3-pyridinecarboxylic acid methyl ester
Ca ²⁺	calcium
cAMP	cyclic adenosine 3',5'-monophosphate
cGnRH-II	chicken GnRH-II
DAG	diacylglycerol
ERK	extracellular signal-regulated kinase
ETYA	5,8,11,14-Eicosatetraynoic acid
FSH	follicle-stimulating hormone
GF109203X	2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide
GfA	goldfish GnRH receptor A
GfB	goldfish GnRH receptor B
GH	growth hormone
GnRH	gonadotropin-releasing hormone
GtH	gonadotropin hormone
H7	1-(5-isoquinolinesulfonyl)-2-methylpiperazine
H89	N-[2-((<i>p</i> -bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide
IP	inositol phosphate

IP ₁	inositol 1-phosphate
IP ₂	inositol 1, 4-bisphosphate
IP ₃	inositol 1, 4, 5-trisphosphate
JNK	c-jun amino-terminal kinase
LH	luteinizing hormone
LTC ₄	leukotriene C ₄
LTD ₄	leukotriene D ₄
LTE ₄	leukotriene E ₄
MAPK	mitogen-activated protein kinase
MEK	MAPK/ERK kinase
MPF	maturation promoting factor
PD98059	2'-Amino-3'-methoxyflavone
PKA	protein kinase A
PKC	protein kinase C
PLA ₂	phospholipase A ₂
PLC	phospholipase C
PLD	phospholipase D
PMA	phorbol-12-myristate-13-acetate
SAPK	stress-activated protein kinase
sGnRH	salmon GnRH
TMB-8	8-(N,N-diethylamino)-octyl-3,4,5-trimethoxybenzoate
U-57908	1,6-bis(cyclohexyloximinocarbonyl-amino)hexane
VSCC	voltage-sensitive calcium channel

Acknowledgements

The authors acknowledge grant support from the Natural Sciences and Engineering Research Council of Canada. C.K. is supported by an Alberta Heritage Foundation for Medical Research studentship.

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Gonadotropin-releasing hormone receptor: cloning, expression and transcriptional regulation

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Introduction

Gonadotropin-releasing hormone (GnRH), also referred to as LHRH (luteinizing hormone-releasing hormone), is a hypothalamic decapeptide (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH₂), which was isolated and characterized by groups led by Schally and Guillemin, the 1977 Nobel laureates. It is synthesized by hypothalamic neurons and released into the portal blood in a pulsatile fashion. GnRH stimulates the synthesis and secretion of gonadotropins (follicle stimulating hormone, FSH; and luteinizing hormone, LH) from gonadotropes in the anterior pituitary. Gonadotropins, in turn, regulate both the gametogenic and steroidogenic functions of the gonads (Fink, 1988). These actions of GnRH are achieved through its high affinity receptors that are expressed on the plasma cell membranes of gonadotropes. The expression of mRNA for GnRH and its receptor in pituitary as well as extra-pituitary tissues, including the placenta, ovary, myometrium, endometrium, breast, prostate, and blood mononuclear cells, has been documented. The binding of GnRH to its specific receptors initiates activation. Ligand binding allows coupling of receptor to the G α_{q11} protein with generation of several second messengers, most notable diacylglycerol

and inositol triphosphate (IP₃). The increased level of diacylglycerol leads to activation of protein kinase C, and the increased level of inositol triphosphate leads to the production of cAMP and the release of calcium from intracellular pools, with both of these downstream events leading to secretion of FSH and LH. Following prolonged, continuous activation of the GnRH receptor by GnRH, however, the release of gonadotropins is followed by receptor desensitization and a cessation of LH secretion. Our understanding of the functioning of GnRH receptor, the molecular mechanisms that mediate its signal transduction, and its transcriptional regulation has been advanced by the cloning of the GnRH receptor cDNA and its gene. In this review, we present a summary of the cloning of the GnRH receptor cDNA and its gene, and recent insights into its regulation.

Molecular structure of GnRH receptors

The cloning of the GnRH receptor has greatly improved our understanding of the mechanisms that underlie GnRH regulation of reproductive functions, as well as other extra-pituitary functions. Using the *Xenopus* oocyte as an expression system, Tsutsumi and colleagues (Tsutsumi et al., 1992) successfully isolated a cDNA clone for the GnRH receptor from a mouse gonadotrope cell line (α T31). As anticipated, the GnRH receptor contains seven transmembrane domains, belongs to the G-protein-coupled receptor family, and utilizes Ca²⁺ as a second messenger. Subsequently, Kakar and others isolated the cDNAs for the GnRH receptors of humans (Kakar et al.,

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1992; Chi et al., 1993), as well as the rat (Kakar et al., 1994a), cow (Kakar et al., 1993), ovine (Brooks et al., 1993), porcine (Weesner and Matteri, 1994), and marmoset (Byrne et al., 1999). Like the mouse receptor, the GnRH receptors from all the species analyzed to date contain seven transmembrane domains and belong to the family of G-protein-coupled receptors.

The deduced amino acid sequence of the human GnRH receptor is shown in Fig. 1. The human, cow, ovine, porcine and marmoset monkey receptor cDNAs encode a protein of 328 amino acids, whereas the mouse and rat receptor cDNAs encode 327 amino acid proteins, as illustrated in Fig. 2. The predicted amino acid sequences for the GnRH receptors of all of the mammalian species are 85% identical. The binding affinity of the cloned receptors after their transfection into heterologous systems is high, and the pharmacologic properties are similar to those of the native receptors (Brown and Reeves, 1983; Wormald et al., 1985; Horn et al., 1991; Tsutsumi et al., 1992). The cloning of GnRH receptors from human breast and ovarian tumors, gonads and placenta demonstrated that the sequences of these receptors are identical to those of the pituitary receptor (Kakar et al., 1994b; Moumni et al., 1994; Boyle et al., 1998).

Analysis of the amino acid sequences of the GnRH receptors revealed homology with other G-protein-coupled receptors, with some amino acid residues in the transmembrane domains 2 (TM2), 3 (TM3), 5 (TM5), 6 (TM6), and 7 (TM7), and the first extracellular domain being highly conserved among this group of proteins (Davidson et al., 1994). Notably, there is a conserved proline residue in each of the TM2, TM4, TM5, TM6 and TM7 domains, and residues Asn⁵³ (TM1), Trp¹⁶⁴ (TM4), Ser¹⁶⁷ (TM4), Asn¹³⁵ (TM7) and Tyr³²³ (TM7) are conserved. There is, however, little sequence homology with other G-protein-coupled receptors in the intra- and extracellular domains of GnRH receptors. The human, cow, ovine and porcine receptor sequences contain two potential sites for N-linked glycosylation (N-X-S/T), one in the amino terminus and one in the first extracellular domain (Fig. 2), whereas the rodent receptors contain three potential N-linked glycosylation sites. Although mutation of the N-linked glycosylation site in the N-terminal domain to Gln caused

a change in apparent molecular weight, the ligand binding affinity for the receptor was not altered. Moreover, alteration of the potential glycosylation site (Asn) in the first extracellular domain to Gln did not affect the mobility of the receptor nor its binding affinity. These results suggest that although glycosylation of GnRH receptors may be essential for receptor expression and stability, it probably is not required for GnRH binding (Davidson et al., 1995). As in many of the G-protein-coupled seven transmembrane receptors, cysteine residues are present in the first and second extracellular domains of GnRH receptors, suggesting the presence of disulfide bonds, which have been reported to stabilize ligand binding (Dohlman et al., 1990). Using site-directed mutagenesis and photoaffinity cross-linking, Davidson et al. (1997) identified two cystine disulfide bridges between Cys¹⁴–Cys²⁰⁰, and Cys¹¹⁴–Cys¹⁹⁶ respectively. Consistent with findings for the human GnRH receptor, Cook and Eidne (1997) reported the presence of two disulfide bridges between Cys¹⁴–Cys¹⁹⁹, and Cys¹¹⁴–Cys¹⁹⁵ for the rat GnRH receptor. These disulfide bridges were proposed to maintain the structure and function of the GnRH receptor (Cook and Eidne, 1997; Davidson et al., 1997). The GnRH receptors also contain five potential sites for phosphorylation. These sites are located in the intracellular loops and may be involved in receptor desensitization and signal transduction (Fig. 1).

There are several features of the GnRH receptor that are unusual in the G-protein-coupled receptor family. These include lack of the entire intracellular C-terminal domain that is required for desensitization of many G-protein-coupled receptors (Attwood et al., 1991). In addition, there are unusual substitutions at loci that are highly conserved, such as modification of the more common DRY sequence, known as the 'signature' sequence, that is localized at the junction of the third transmembrane domain and the second intracellular loop. DRY is replaced by DRS in GnRH receptors (Fig. 1). Mutation of DRS to DRY in the mammalian receptors resulted in a small increase in agonist affinity with no discernible change in signal transduction (Arora et al., 1995). Finally, there is a reciprocal interchange of Asn⁸⁷ and Asp³¹⁸ residues in transmembrane domains 2 and 7 as compared to the G-protein-coupled receptors.

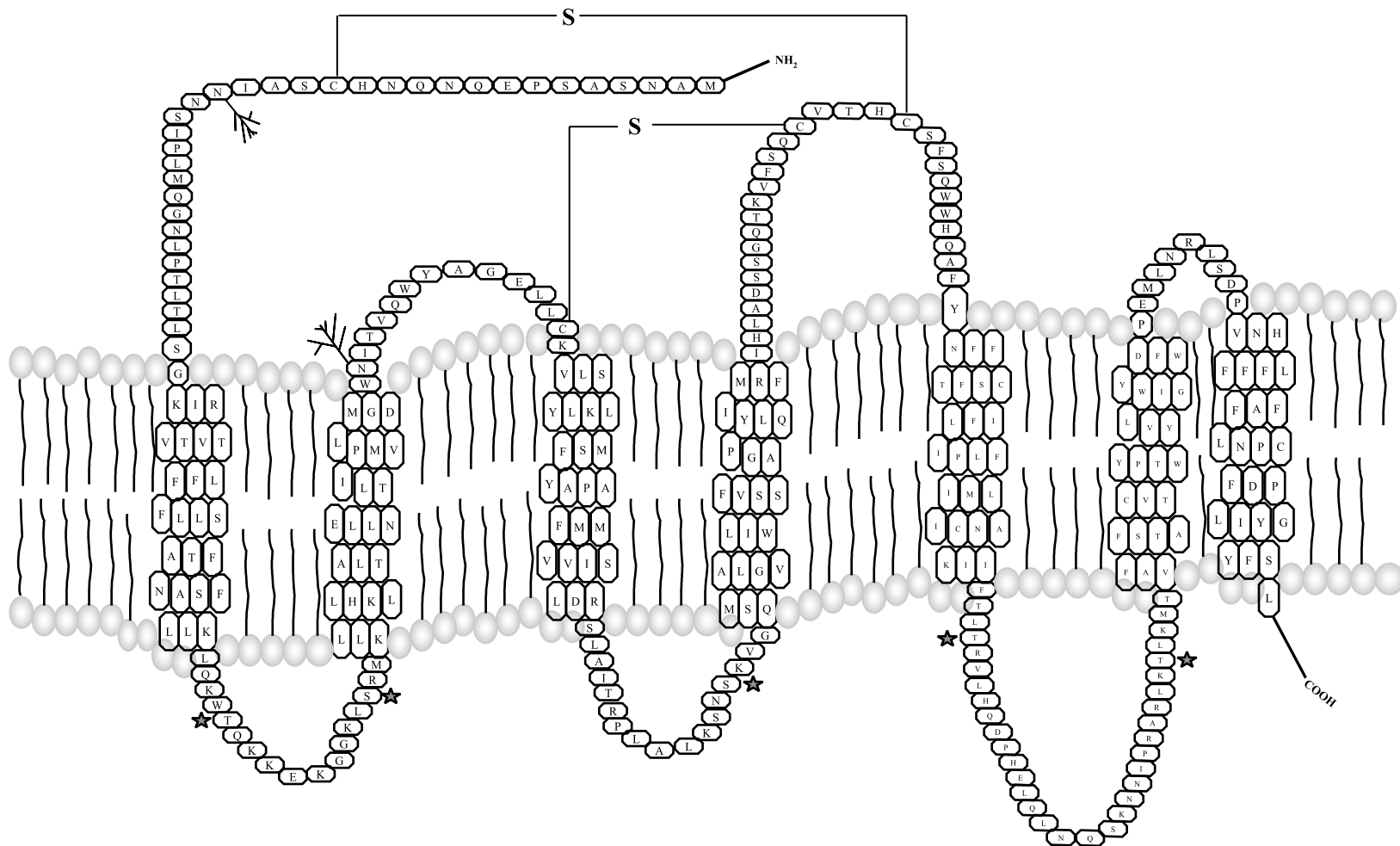


Fig. 1. Model of the human GnRH receptor. The receptor is shown in proposed seven transmembrane topology. The potential sites for N-linked glycosylation (N), phosphorylation by protein kinase C (★) and disulfide bridges between cysteine residues (-S-) are indicated.

bGnRHR	1	MANSDSPEQNE	NHCSAINS	SIPLTPGS	LPPTLTLSGKIRVTVTFFFLFLLST	I	FNT	SFLLKL
OGnRHR	1	MANGDSPNQNE	NHCSAINS	SIPLTPGR	LPPTLTLSGKIRVTVTFFFLFLLST	I	FNT	SFLLKL
pGnRHR	1	MANSASPEQON	NHCSAINS	SIPLTQGN	LPPTLTLSGKIRVTVTFFFLFLLST	A	FN	SFLLKL
hGnRhR	1	MANSASPEQON	NHCSAINN	SIPLMQGN	LPPTLTLSGKIRVTVTFFFLFLLS	A	FN	SFLLKL
mGnRHR	1	MANNASLEQD	PNHCSAINN	SIPLIQGK	LPPTLTLSGKIRVTVTFFFLFLLST	A	FN	SFLLKL
rGnRHR	1	MANNASLEQD	QNHCSAINN	SIPLTQGK	LPPTLTLSGKIRVTVTFFFLFLLST	A	FN	SFLLKL
bGnRHR	61	QNWTOQRKEKR	KKLSRMKLL	LLKHLTLANLLET	LIVMPLDGMWNITVQWYAGE	L	LCKVLSYL	
OGnRHR	61	QNWTOQRKEKR	KKLSKMKVLL	LLKHLTLANLLET	LIVMPLDGMWNITVQWYAGE	L	LCKVLSYL	
pGnRHR	61	QKWTQQRKEK	GKKLSRMKVLL	LLKHLTLANLLET	LIVMPLDGMWNITVQWYAGE	F	LCKVLSYL	
hGnRhR	61	QKWTQQRKEK	GKKLSRMKLL	LLKHLTLANLLET	LIVMPLDGMWNITVQWYAGE	L	LCKVLSYL	
mGnRHR	61	QKWTQQRKKG	GKKLSRIK	VLLKHLTLANLLET	LIVMPLDGMWNITVQWYAGE	F	LCKVLSYL	
rGnRHR	61	QRWTQQRKKG	GKKLSRMKVLL	LLKHLTLANLLET	LIVMPLDGMWNITVQWYAGE	F	LCKVLSYL	
bGnRHR	121	KLFSMYAPAFMM	VVISLDRSLAIT	KPLAVKSNSKLGQ	FMIGLAWLLSS	I	FAGPQLYIFGM	
OGnRHR	121	KLFSMYAPAFMM	VVISLDRSLAIT	RPLAVKSNSKLGQ	FMIGLAWLLSS	I	FAGPQLYIFGM	
pGnRHR	121	KLFSMYAPAFMM	VVISLDRSLAIT	RPLAVKSNSRLGR	FMIGLAWLLSS	I	FAGPQLYIFRM	
hGnRhR	121	KLFSMYAPAFMM	VVISLDRSLAIT	RPLALKSNSKVGQSM	VGLAWLLSS	V	FAGPQLYIFRM	
mGnRHR	121	KLFSMYAPAFMM	VVISLDRSLAIT	QPLAVQSNSKLEQSM	ISLAWLLS	I	VFAGPQLYIFRM	
rGnRHR	121	KLFSMYAPAFMM	VVISLDRSLAVT	QPLAVQSKSKLERS	MTSLAWLLS	I	VFAGPQLYIFRM	
bGnRHR	181	IHLADDSGQTEG	FSQCVTHCSFPQ	WWHQAIFYNFFTF	SCLFIIPLLIMV	I	CNAKIIFTLTR	
OGnRHR	181	IHLADDSGQTEG	FSQCVTHCSFPQ	WWHQAIFYNFFTF	SCLFIIPLLIML	I	CNAKIIFTLTR	
pGnRHR	181	IHLADSSGQTEG	FSQCVTHGSPQ	WWHQAIFYNFFTF	SCLFIIPLLIML	I	CNAKIMFTLTR	
hGnRhR	181	IHLADSSGQTKV	FSQCVTHCSFS	QWWHQAIFYNFFTF	SCLFIIPLLF	I	MICNAKIIFTLTR	
mGnRHR	181	IYLDGSG-PTV	FSQCVTHCSFPQ	WWHQAIFYNFFTF	GCLFIIPLLIML	I	CNAKIIFALTR	
rGnRHR	181	IYLVVDGSG-PAV	FSQCVTHCSFPQ	WWHQAIFYNFFTF	SCLFIIPLLIML	I	CNAKIIFALTR	
bGnRHR	241	VLHQDPHKLQ	LNQSKNNIPRAR	LRTLKMTVAFATS	SFTVCWTPYYVL	G	IWYWFDPDMVNRV	
OGnRHR	241	VLHQDPHKLQ	LNQSKNNIPQ	ARLRTLKMTVAFATS	SFTVCWTPYYVL	G	IWYWFDPDMVNRV	
pGnRHR	241	VLQDPHKLQ	LNQSKNNIPRAR	LRTLKMTVAFAT	ASFIVCWTPYYVL	G	IWYWFDPDMVNRV	
hGnRhR	241	VLHQDPHKLQ	LNQSKNNIPRAR	LRTLKMTVAFATS	SFTVCWTPYYVL	G	IWYWFDPDMVNRV	
mGnRHR	240	VLHQDPRKLQ	LNQSKNNIPRAR	LRTLKMTVAFATS	SFVVCWTPYYVL	G	IWYWFDPDMVNRV	
rGnRHR	240	VLHQDPRKLQ	LNQSKNNIPRAR	LRTLKMTVAFCT	SFVICWTPYYVL	G	IWYWFDPDMVNRV	
bGnRHR	301	SDPVNHFFFL	FAFLNPCFDPLI	YGYFSL				
OGnRHR	301	SDPVNHFFFL	FGFLNPCFDPLI	YGYFSL				
pGnRHR	301	SDPVNHFFFL	FAFLNPCFDPLI	YGYFSL				
hGnRhR	301	SDPVNHFFFL	FAFLNPCFDPLI	YGYFSL				
mGnRHR	300	SEPVNHFFFL	FAFLNPCFDPLI	YGYFSL				
rGnRHR	300	SEPVNHFFFL	FGFLNPCFDPLI	YGYFSL				

Fig. 2. Comparison of the deduced amino acid sequence of the bovine (b), ovine (o), porcine (p), human (h), mouse (m) and rat (r) GnRH receptors. Amino acids are represented by single letter codes. Identical amino acids are boxed. Amino acid present in bovine, ovine, porcine and human GnRH receptors but absent in mouse and rat GnRH receptors is indicated by hyphen (—).

Cloning of GnRH receptors from non-mammalian species

To date at least 12 forms of GnRH have been identified in vertebrates (Sherwood et al., 1993, 1997; Craig et al., 1997; King and Millar, 1997) and 2 variants of GnRH in an invertebrate, the tunicate (Powell et al., 1996). Two or more forms of GnRH are present in most vertebrate species (Sealfon et al., 1997). One form is represented by mammalian

GnRH and its non-mammalian counterparts, which have a predominant function as neuropeptides regulating the pituitary. The second form of GnRH, first identified in chicken brain (His⁵Trp⁷Tyr⁸GnRH), is the most ubiquitous form in vertebrates, and most species have this form together with one or two other GnRHs. The mammalian GnRH (GnRH-I) exhibits high affinity binding to mammalian GnRH receptors but only low affinity binding to the non-mammalian receptors. On the other hand, non-

mammalian GnRH (GnRH-II, also called chicken GnRH-II) binds non-mammalian GnRH receptors with high affinity and binds mammalian receptors with low affinity. This preferential binding raised the question as to whether a distinct GnRH receptor is expressed in non-mammalian vertebrates. To address this issue, Tensen et al. (1997) cloned the GnRH receptor from African catfish, and subsequently others reported the cloning of receptors from chicken (Troskie et al., 1997), frog (Wang et al., 2001), goldfish (Illing et al., 1999), and *Xenopus* (Troskie et al., 2000). In addition, existence of more than one GnRH receptor subtype has been reported in goldfish (Illing et al., 1999) and frog (Wang et al., 2001). These studies revealed that the GnRH receptors from non-mammalian species encode proteins of 368 to 412 amino acids, which, like mammalian GnRH receptors, contain seven transmembrane domains and belong to the family of G-protein-coupled receptors. The non-mammalian GnRH receptors were found to be only 38% to 41% homologous with the mammalian GnRH receptors at the amino acid level. Unlike mammalian GnRH receptors, non-mammalian GnRH receptors contain the C-terminal domain of 51 amino acids. The significance of this C-terminal domain of the non-mammalian GnRH receptors in the desensitization and internalization of the receptors was demonstrated by Pawson et al. (1998), who showed that deletion of the C-terminal domain from the chicken GnRH receptor resulted in a reduction in the internalization of the receptor. Furthermore, fusion of the C-terminal tail of the catfish GnRH receptor to the rat receptor resulted in an increase in receptor desensitization and internalization (Lin et al., 1998), suggesting that the C-terminal tail is essential for rapid receptor desensitization and internalization.

Cloning of mammalian GnRH receptor II

As discussed above, the existence of GnRH-II (chicken GnRH-II) in non-mammalian species is well established. More recently, several groups have identified a second form of GnRH (GnRH-II) in the brain of mammalian species (Kasten et al., 1996; Lescheid et al., 1997; Chen et al., 1998; White et al., 1998; Urbanski et al., 1999; Latimer et al., 2000), and the GnRH-II gene was cloned from human

(White et al., 1998) and monkey brain (Urbanski et al., 1999). As GnRH-II receptors exist in non-mammalian species, the search for similar receptors in mammalian species was undertaken, and recently, Neill et al. (2001) and Millar et al. (2001) reported the cloning of GnRH-II receptors from rhesus monkey pituitary, African green monkey kidney cell line (COS-7), and marmoset pituitary. The GnRH-II receptors (GnRH receptor II) that have been cloned from these species encode a protein of 379 amino acids and, as anticipated, belong to the family of G-protein-coupled receptors. However, the GnRH receptor II exhibits only 41% identity with the human type I receptor, and unlike the type I receptor, has a C-terminal tail (Fig. 3). Moreover, the type II receptor is highly selective for GnRH-II. In common with the type I receptor, it couples to $G\alpha_{q11}$ and activates extracellular signal-regulated kinase (ERK1/2) but differs in that it activates p38 mitogen-activated protein (MAP) kinase.

The expression of this GnRH receptor II in humans remains controversial, however. It has been reported that exon 3 transcripts can be found in various human tissues (Millar et al., 2001; Neill et al., 2001), but the existence of a full-length transcript have not been described to date. A sequence homologous to the monkey GnRH receptor II has been localized on human chromosome 1, and a truncated complementary sequence homologous to ribosomal protein RBM8A has been localized on human chromosome 8 (Millar et al., 1999; Faurholm et al., 2001). Based on the genomic sequence, Faurholm et al. (2001) predicted that the human GnRH receptor II does not possess a classical methionine as the start codon but does contain a stop codon in exon 2, suggesting that the human GnRH receptor II may be truncated and non-functional.

Cloning of the human GnRH receptor gene and its molecular structure

Using PCR analysis of the somatic hybrid cell lines and chromosomal *in situ* hybridization, we localized the human GnRH receptor gene to chromosome 4 (4q13.1) (Kakar and Neill, 1994). The mouse gene has been mapped by linkage analysis to be within 1.2 ± 1.2 centimorgans of the chromosome 5 marker Pmv-11 (Kaiser et al., 1994), and the ovine gene

hGnRHRI	-MANSASPEQNQNHC SAINNSIPLMQGN-LPTLTLSGKIRVTVTFFLFLLSATFNASFL	58
mGnRHRII	MSAGNGTPWGSAAAGEESWAASGVAVEGSELPTFSAAAKVRVGVTVIVLVSSAGGNLAVLW	60
hGnRHRI	KLQKWTQKKEKGKLSRMKLLKHLTLANLLETIVMPLDGMWNITVQWYAGELLCKVLS	118
mGnRHRII	SVTRPQPSQLRP---SPVRTLFAHLAAADLLVTFVVMPLDATWNIIVQWLAEDIACRTLM	117
hGnRHRI	YLKLFMYAPAFMMVVISLDRSLAITRPLALKSNSKVGQSMVGLAWILSSVFAGPQLYIF	178
mGnRHRII	FLKLMAMYSAAFLPVVIGLDRQAAVLNPLGSRSGVRK---LLGAAWGLSFLALPQLFLF	174
hGnRHRI	RMIHLADSSGQTKVFSQCVTHCSFSQWWHQAFFYNFFTFSCFLIIPLFIMLICNAKIIFTL	238
mGnRHRII	HTVHRA---G-PVPFTQCVTKGSEKARWQETTYNLFTFRCLFLLPLTAMAICYSHIVLSV	230
hGnRHRI	----TRVL-HQDPHELQLNOSKNNIPRARKTLKMTVAFATSFTVCWTPYYVLGIWYWFD	293
mGnRHRII	SSPQTRKGSHPAGEFALCRSFDNCPVRWLWALRLALLILLTFILCWTPYYLLGLWYWF	290
hGnRHRI	PEMLNRLSDPVNHFFFLFAFLNPCFDPLIYGYFSL*	328
mGnRHRII	PTMLTEVPPSLSHILFLFGLLNAPLDPLLYGAFTLGCQRGHQELSIDSSENEGSGRMLQQE	350
mGnRHRII	IHALRQQEVQKTVTSTRSAGETKDISITSI*	379

Fig. 3. Comparison of the deduced amino acid sequence of the human GnRH receptor type I (hGnRHRI) and monkey (*Macaca mulatta*) GnRH receptor type II (mGnRHRII). The amino acids are represented by single letter codes. Identical amino acids are boxed. Gaps (-) are generated to obtain maximum alignment.

was localized near the *FecB* locus on chromosome 6 (Montgomery et al., 1995). The structures of the human, mouse, rat and ovine GnRH receptor genes have been characterized (Albarracin et al., 1994; Fan et al., 1995; Campion et al., 1996; Kakar, 1997; Reinhart et al., 1997). A comparison of the GnRH receptor gene sequences and cDNA sequences reveals that, unlike other G-protein-coupled receptors that are intronless, each of these genes has two introns at the same locations in the reading frame. The first intron (intron 1) is larger in the mouse (15 kb) than in the rat (12 kb), human (4 kb), or sheep (>10 kb). It is located in transmembrane domain 4, with intron 2 being located between transmembrane domains 5 and 6. In the human gene, exon 1 encodes the 5' untranslated sequence and nucleotides +1 to +522 in the open reading frame; exon 2, encodes nucleotides +523 to +742, and exon 3, encodes nucleotide +743 to +983 in the open reading frame and the 3' untranslated sequence (Fig. 4). The human exon 2 is three nucleotides longer than the mouse and rat genes, resulting in a single additional amino acid (Lys¹⁹¹) (Fig. 2) in the second extracellular loop of the human receptor. There is 42% sequence ho-

mology among the genes of all four species within a 0.9 kb region from the ATG codon. The human GnRH receptor gene is more closely related to the ovine gene with 70% sequence homology, whereas same region of the human gene is 46% homologous with the rat and mouse genes (Campion et al., 1996). One significant difference between the mouse, rat, and ovine genes, and the human gene is the location of the transcriptional start sites. The start sites for the rat and mouse are clustered in a region within 110 bp from the translation codon (ATG) except the distal site described by Clay et al. (1995). Mapping of the transcription start site(s) for the human GnRH receptor using 5' extension and 5'-RACE analysis of human pituitary RNA revealed multiple transcription start sites in the 5' flanking region of the gene. Fan et al. (1995) using human brain RNA reported five transcriptional start sites, whereas, using human pituitary RNA, we found 18 transcriptional start sites (Figs. 5 and 6) (Kakar, 1997). The nearest start sites in the human and ovine genes are at least 600 bp upstream from the ATG start codon. The human GnRH receptor gene contains multiple TATA sequences (six compared with none in mouse) and

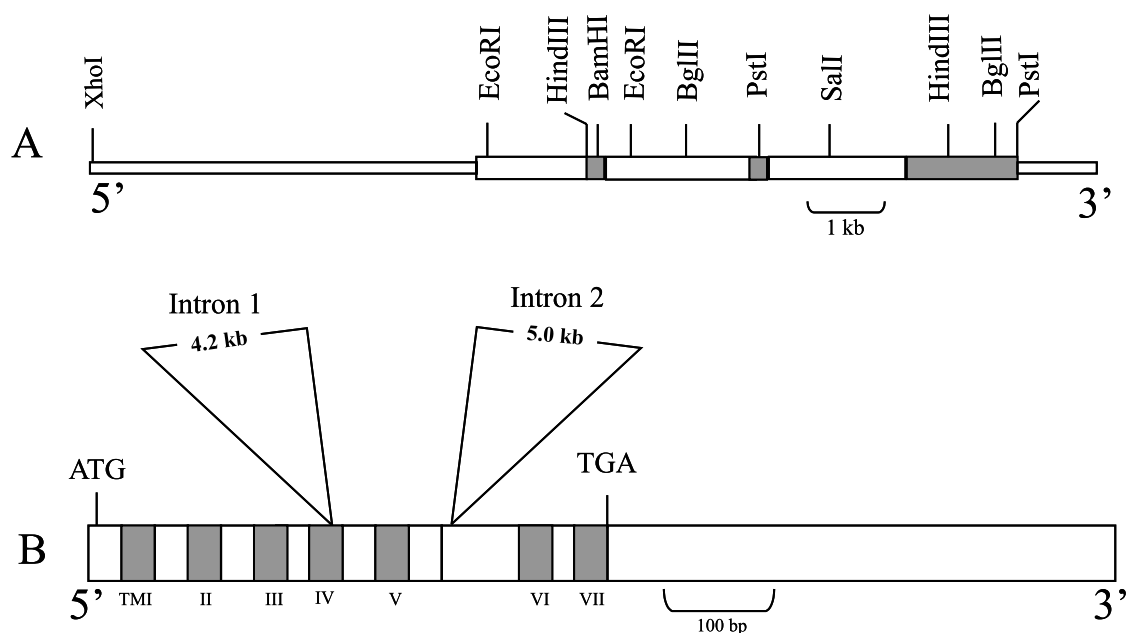


Fig. 4. Genomic organization of the human GnRH receptor gene. Panel A: The solid boxes indicate the exons. The relevant restriction enzymes sites are shown. Panel B: The structure of the human GnRH receptor cDNA. Open box indicate the coding region. Solid boxes indicate the putative transmembrane domains. The location and sizes of the introns are shown. Reproduced from Kakar (1997) with permission.

CAAT sequences (six) distributed over a 669 bp region from ATG (Fan et al., 1995; Kakar, 1997). The longest transcript has been confirmed by PCR and represents a transcript of 1393 bp of 5'-untranslated sequence (Fan et al., 1995). The existence of multiple transcription sites and the differences in these sites in the human pituitary and brain suggest the possibility of alternate transcriptional start sites in the pituitary and differences in the regulatory mechanisms in the pituitary and extra-pituitary tissues. In the human GnRH receptor gene, with regard to transcriptional regulation, the cAMP response element (CRE) has been identified at -2191 (relative to transcription start codon; ATG), and a putative glucocorticoid/progesterone response element (GRE/PRE) with one base pair replacement differentiating it from a functional GRE/PRE is found at -793 (relative to translation start codon). Additionally, AP-1 and AP-2 binding sites that implicate the effect of kinase C on the regulation of the receptor gene have been identified at -1517 and -4570 (relative to translation start site), respectively (Figs. 5 and 6). Pit-1, an anterior pituitary-specific transcrip-

tion factor (Imagawa et al., 1987), which has been reported to be essential to the expression of growth hormone and prolactin (Bodner et al., 1988; Ingraham et al., 1990), and a steroidogenic factor-1 (SF-1) binding site were identified at -942 and -134 (relative to translation start site), respectively (Fan et al., 1995; Kakar, 1997; Ngan et al., 1999). At the 3' end of the gene, five polyadenylation signals are found, which are distributed over 800 bp (Fan et al., 1995).

Determination of the putative promoter for the GnRH receptor gene

To identify the promoter sequence for the human GnRH receptor gene, we analyzed a 4600 bp sequence upstream of the translation start site (ATG), and prepared a number of pGL3-luc (luciferase) chimeric constructs containing the gene sequences (-4615 to +1), (-4615 to -1018), (-4115 to -1018), (-3571 to -1018), (-2284 to -1018), (-2284 to -1657), (-2106 to -1657), (-2003 to -1657), (-1350 to +1), (-936 to +1), (-936 to -397) and (-811 to -215) (relative to trans-

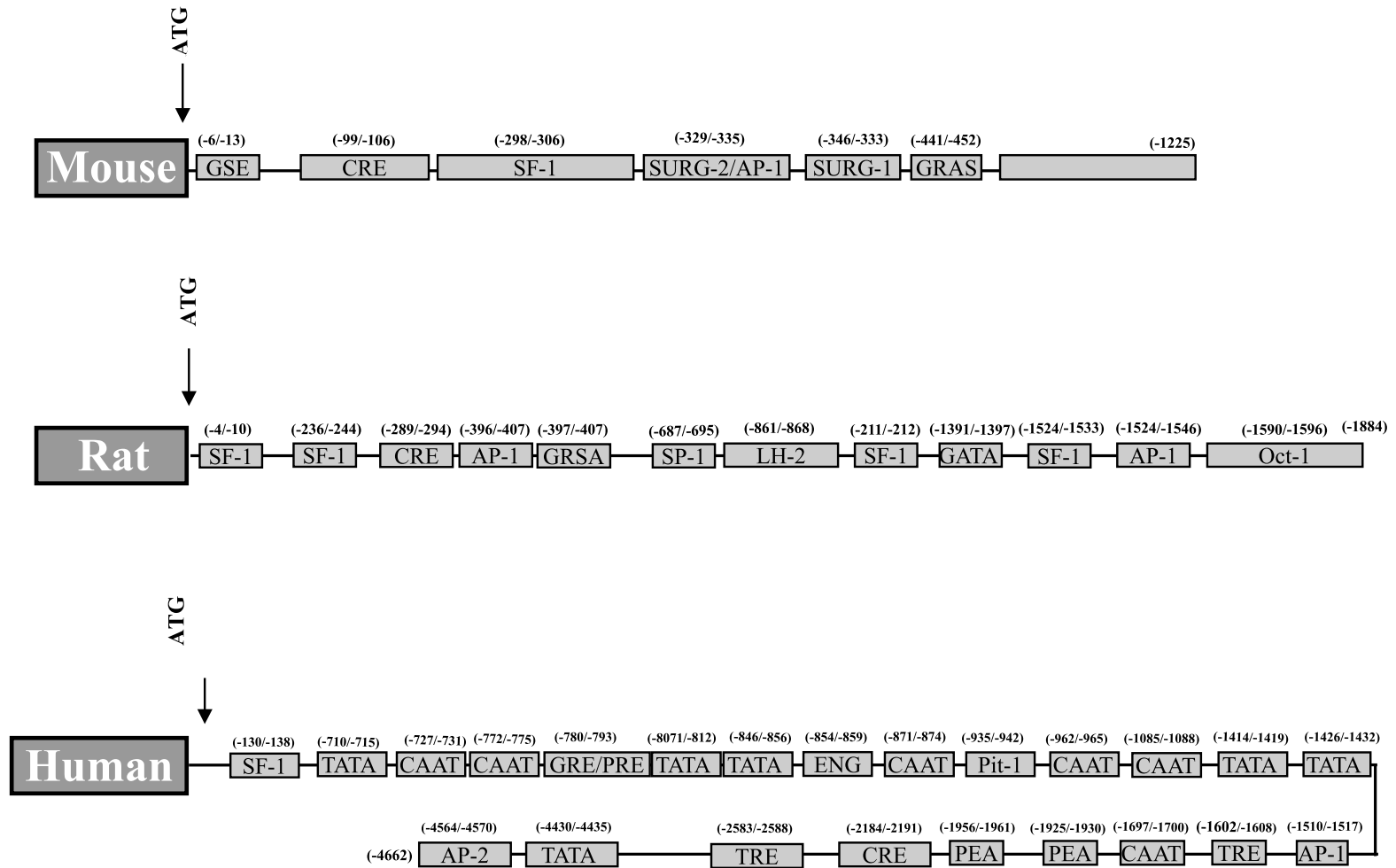


Fig. 5. Representations of the mouse, rat and human GnRH receptor gene 5' flanking region. The translation start site is indicated by arrow (ATG). The regulatory element sequences are boxed and indicated. The numbering shown is relative to translation start site (ATG; +1).

lation start site). These constructs along with a pSV-gal (galactosidase) plasmid were cotransfected into a mouse pituitary gonadotrope-derived cell line (α T31), a human endometrial tumor cell line (HEC-1A), and an African green monkey kidney cell line (COS-7). After 48 h of transfection, the cells were lysed and luciferase activity was assayed. Based on this activity, we identified two putative promoters and one negative region for the gene. The distal promoter resides between -2003 and -1657 and the proximal promoter resides between -811 to -397 , whereas a suppressor resides between -936 to $+1$ (unpublished results). Consistent with our results is the report of Kang et al. (2000) who, using similar techniques and α T31 and an ovarian tumor cell line (OVCAR-3), identified two putative promoters and a negative regulatory region. They localized one promoter, designated PR1, to a region between -771 to -557 , and, a second, PR2 between -1351 to -1022 , with a negative control region residing between -1022 to -771 in both the pituitary and ovarian cells. These investigators further reported the utilization of the same promoters to drive the basal promoter activities in both α T31 and OVCAR-3 cell, suggesting that the usage of the human GnRH receptor gene is not cell-specific. Ngan et al. (2000), using 5' and 3' deleted constructs of the human GnRH receptor gene flanking region, identified a distal promoter element at $-1705/-1674$ with reference to start codon (ATG). This promoter was found to be immediately 5' to a previously identified CAP site at -1673 in the human pituitary gene. In addition, these investigators identified a pyrimidine-rich initiator element (Inr) (-1682) and a CAAT box (-1702) within the promoter sequence, and mutation of these elements abrogated both promoter activity and nuclear protein binding to the promoter sequence, suggesting that these elements may play an important role in regulating promoter activity. On the other hand, Cheng et al. (2000a), using a human placental cell line (JEG-3), reported the existence of promoter sequence between -1737 and -1346 , relative to the translation start site. Taken together, these studies suggest the existence of a number of different promoters for the human GnRH receptor gene, and it is tempting to speculate that these differences may be associated with tissue-specific expression of the human GnRH receptor gene. It remains unclear,

however, as to which promoters and transcriptional start sites are utilized in different tissues in particular in pituitary and tumors.

In their analysis of the 1.2 kb 5' flanking region of the mouse GnRH receptor gene, Albarracin et al. (1994) and Clay et al. (1995) showed a high level of promoter activity in α T31 cells but low levels of promoter activity in a rat somato-gonadotrope cell line (GH3), a human choriocarcinoma cell line (JEG-3), a monkey kidney fibroblast cell line (CV-1), and a monkey kidney cell line (COS-7). These findings support the concept of pituitary-specific promoter activity. In further studies, Clay et al. (1995), Duval et al. (1997a), Norwitz et al. (1999) and White et al. (1999) identified a 500 bp region that supported basal and GnRH-stimulated expression of the mouse GnRH receptor gene. Similarly, a rat 5' flanking region has been reported to be associated with high promoter activity in gonadotrope α T31 cells but not in somatotrope GH3b, Chinese hamster ovary CHO, and monkey kidney COS-7 cells (Reinhart et al., 1997; Pincas et al., 1998). Again, this cell-specific expression of the GnRH receptor gene indicates the presence of tissue-specific or cell-specific transcriptional regulation of both the rat and mouse GnRH receptor genes. In contrast, however, the human GnRH receptor gene promoter(s) has been found to have equivalent activity in pituitary cells and extra-pituitary cells (unpublished results), a finding which is consistent with our previous finding for the expression of GnRH receptor mRNA in pituitary and extra-pituitary tissues (Kakar and Jennes, 1995). The level of human GnRH receptor promoter activity was much lower in α T31 cells when compared to that of the rat and mouse receptor promoters, which may be due to the lack of specific factors in the mouse gonadotrope cell line (α T31) (Cheng et al., 2000a; Ngan et al., 2000).

Transcriptional regulation of the GnRH receptor gene

The concentration of the GnRH receptor protein is regulated tightly and exhibits both up- and down-regulation in response to its cognate ligand, gonadal steroids and peptide hormones (Clayton and Catt, 1981a,b; Lloyd and Childs, 1988; Laws et al., 1990; Gregg et al., 1991; Kaiser et al., 1993; Kakar et al.,

-4662	gcctggggcaa	tatggtgsss	ccctgtctct	actaaaaata	caaaaaattag	ctgtgcatgg
				AP-2		
-4602	tgggtgcacac	ctgttagtccc	agctacttgg	gaggctgagg	cgaagaagacc	acttgaaccc
-4542	aggaaggcga	aggttgcagt	gagccgagat	ctgtgccactg	cactccagcc	tgggcgacag
					TATA	
-4482	agcaagactc	ttgattgaaa	aaaaaattca	taacaataca	atataagtat	aaaatatggt
-4422	tggaaatatc	atatacgagc	tttggctagc	cttatTTTTTg	“tatccacaa	gtatagtgtg
-4362	tagtacataa	tgtttaataa	gtgggttaatt	gaattgttga	aataaatata	attcttatat
-4302	tcctgggttta	aatctTTTTT	tatatgttta	attatactta	aaagtaaaga	ttactacttt
-4242	tagtatTTTg	tgaacataaa	ttttgataag	taaacatact	tatttatgct	ctgcttttct
-4182	gttcatttca	tatggttggtg	atttatatta	tatatgattt	ccttaactctg	atctccaaaa
-4122	tcgtacatat	ttaacataaa	tttcccatca	tttactctta	agacatctat	actgaaataa
-4062	aaattgcaac	aatgtttttac	agtgtgattc	tccaaataat	gagcacttct	tgggaataatg
-4002	aaagtgaatc	tgccatgtta	aatgtatgca	taatttaaca	tggcttagaa	aaatttgtat
-3942	ggctttttaa	atttgtttgct	ctccttcaca	acaattttgt	gtatatattt	cctcatacta
-3882	cttctaccac	agtccttctct	caaatgtctc	catttaaaact	taaaattttaa	aagttccccct
-3822	tttactcatt	tcacagttgt	cgtagacact	atcattcttt	gctcaacaaa	gtaagtcatg
-3762	ttttcaaaaa	ctaaaaaaa	aaaaattctt	ttttttctga	tatttgtcta	cttccatctt
-3702	ctctccttac	aggaacaaaa	attttttccc	aaatacattt	gcatacacat	tcttcttatt
-3642	tgaaagactg	ccttctctta	ttatttttatt	ctcatgagta	aagaagtatg	aaaaatattt
-3582	cttgtctccc	tgggtcactt	ctaaaccatc	atcactcttt	ccttcagaag	ttggacttct
-3522	aattcaaaaa	aaaaaaaaaa	acttggctag	ttaggttcaa	gaagatgatg	ggaaaaaacac
-3462	tatgaagaaa	aaaaagatac	tataattgaa	aaccaactca	ggtgatgaat	cagcaggagt
-3402	tgtctatcct	taaagctgcc	tgggtcctctg	ttggctttct	aggtcttggtg	tatcatgaaa
-3342	gataactcca	ttatctaaaa	cagtcctcct	ttcctgcaac	taaaagggcc	tgttaacacg
-3282	gcccttctatt	cactaaggac	tatggcacct	ggctcataca	cctttccacc	ccaactgcta
-3222	ccagaaagta	gcattgcata	gtagttaaag	acaggagctc	tgaaggcaga	ctgcatgaga
-3162	gttaacacaa	ttctgtgggtg	tcctaatttag	ggaaaaggag	tcaggctggt	gggagcaggg
-3102	gaaagcaaaa	agaaaaagca	gataagctac	aaggacacat	agccctctctg	tgcaaaataac
-3042	tctcaatctt	cctatgccca	actatcacca	gacacctgca	agttagctca	ctgcaacctt
-2982	ggcattatca	gtactgcaca	aagccctctt	cagcatacag	cataaacact	atcctataaa
-2922	atctccagca	agccttttgt	tctttgcagt	cagcttccct	tccgctgata	ctgcccattg
-2862	tctccctggc	aacgtatttt	ctctccttct	ctaacaaatt	tgcctttctt	ttactcaaaa
-2802	tggtaaaccc	ttttgtccct	gcacctcggg	cttagatagc	caccgctccc	catgacaaac
-2742	tctaatatgg	atcaggtatg	taaattctct	ctgcttcacc	ttcctcaacc	ataagacgaa
-2682	aataatagta	ctgacttcta	gggttggtgt	aaggtctaaa	tgtgttaata	cgtgtaacat
				TRE		
-2622	gcttagaaaa	gtgctgccac	acaaagtagt	acccaataaa	tattaactat	gagtaaatat
-2562	tatcagtatt	gacagcacct	tcaaaatcta	tatagttgac	tcatccaaca	acttagattc
-2502	aacaacttag	agctcctcaa	atgcttcaaa	catcattcgc	cctctacttc	ctctaccgac
-2442	tttcatagcc	acaccctgca	tttgagggtc	ttataccttt	catgtttgtg	tgatagcaag
-2382	ggttttccaa	atagattaac	ctccctgaat	tttgattgcc	cactatttcc	caaatccctt
-2322	caaggcatte	tctgtatgct	acacaataag	ctttctgaag	cataaatctg	gccataccta
-2262	cgtatatta	ctccattctt	tatataggtta	aagcctaaac	tctttttctt	ggaatatagg
				CRE		
-2202	ctctaaagat	ctgaagtctg	cctaattatt	tacacttttg	ctttcacata	ccctttgaac
-2142	tttctcacat	tgtcttcgtg	tttgcattgt	ctgctccagc	ttctaagcat	gccctccttg
-2082	tctctatacc	ccattcccca	gccacttatt	acgtataatg	ctgtagtccg	attcagctcg
-2022	gttgcaacct	cttccctaatt	gaatcagtc	atcataaaca	aagaaaaggg	gggagggagg
	PAE-3			PAE-3		
-1962	gaggaagaga	ggaatgaaag	gaggaaaagg	gaagggaagg	gaagggaagg	ggaagggaag
-1902	gggaagggaa	gggaagggga	aggggaaggaa	tgggaggaaa	agggacaaat	aatgaatgat
-1842	atgctctaatt	ctttttcccc	tagatataga	agacsaagaa	gcaaaatata	cttcaactaa
				↓		
-1782	ttgattttta	cataaaatttt	ctttcctttg	ttttttgggt	gctgggtccac	ttacaaacac
			CAAT			↓
-1722	ttttcatatt	tgtatgtctt	tcctaattggt	atcctgtttt	gttcattttca	ggcatatggg

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-1662 cctgatcaga ttaactgaca tgatgtatat gcaaagcctt ttgagttctt cagaaaaata TRE
-1602 aattatctta ttcaagactg attgcttata aggaacttat tatagctaat atagtaggca
-1542 caattttttt tgtaattctc ctagatgagt cagaacttag ttttgatgta ggtaaaaatt AP-1
-1482 ttatggtcac aaatctcagg tgtgagaaaa tctctttcct tgatactcta TATA
-1422 ggatataaat atttcaagtc tggaagtagt gagagaagct ggaattcttg gacatatagt
-1362 gacagtcaaa aaggagctca ggtacaggac tggcttaagc tgrtcaagat tcaggagaca
-1302 gccagtacac agagaagctg aggaaataat acagatatat ctaa acact tatctaacct
-1242 tctgtggtaa caagctcctt aaaggggctg gatgatgttg tgttcacttt ttatcaccag
-1182 caaaggctaa gataatgtat atagtaataa tttagtaacc atttattaaa taataaata
-1122 ttttaagacag aataaacaag tataataaat gaaccaataa gaatgcacca tctaagtcaa CAAT
-1062 aatagccact tttatcctta acattgtacc tgctttggct gctgcagaag caaacttggt
-1002 ggcattagac aaatcaagct ggtgatttaa taaattccaa tgtaagtctt accagtattg CAAT
-942 atgaataact atccagcact caccatgaaa gttaaagaag caacacagaa aaagttccta Pit-1
-882 agtgggtcca atttgaaatg atcagataac ctataaaaga acatattcat attatactaa CA
AT ENG TATA
-822 cataaacaca tataaatgca cttacagcag ttacacagta ttctcttcaa taactagttt GRE/PRE CAAT
-762 ccttatgcat taatgtgtaa taacagcaac tacaatat tt agataattat TATA
-702 gcaataat tt aaaaactgat taaccgtttt actctaactt aagcatggat tggatcagta
-642 agattgatta ataaatttga atgcagtcag ttggattgat tctaatttaa agttttaatt
-582 tgtttagtaa taattttaag tgaatatatt tgtccagtgt tcgagtgtc aacagtgtgt
-522 ttgaaaagga aaacaaagaa tgttttgaga atgtgttaat tccttaagac aatggatttt
-462 aattggatct gttgttttca tttttcttca ttatcattat acatctgtat gttggacaga
-402 acactaacac tgaatatgtt ttagaaagtg ttttttgaag ttattttaat cataatatca
-342 tgactgactt ttgaattcaa aattaggctg tgactatcct tcttcactta ggaagagtgt
-282 tgtgaaagcc agaccatctg ctgaggtgct acagttacat gtggccctca gaatgcgttt
-222 ggctgctct gtttttagcac tctgttggat taccaatata caaaacaagt taacctttga
-162 tctttcacat taagtatctc agggacaaaa tttgacatac gtctaaacct gtgacgttc
-102 catctaaaga aggcagaaat aaaacatgga ctttagattc gggtacaata aaatatcaga
+1
- 42 tgcaccagag acacaaggct tgaagctctg tcctgggaaa atATGGCAA CAGTGCCTCT

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Fig. 6. Nucleotide sequence of the 5' flanking region of the human GnRH receptor gene. Transcription start sites are shown by arrows. Translation start site (ATG) is capitalized and bolded. The TATA box, CAAT box and other regulatory sequences are shaded. The sequence shown is retrieved from GenBank (accession number AF001950).

1993, 1994a; Fernandez-Vazquez et al., 1996). One mechanism by which the level of the GnRH receptor protein is regulated is through changes in the level of GnRH receptor mRNA (Kaiser et al., 1993; Smith and Reinhart, 1993; Brooks and McNeilly, 1994; Kakar et al., 1994a; Fernandez-Vazquez et al., 1996), suggesting regulation of the protein at the transcrip-

tional level. The half-life of the GnRH receptor mRNA has been estimated to be approximately 21 h (Cheon et al., 2000). However, no studies of its regulation have been performed.

A number of regulatory sequences in the rat, mouse and human receptor genes have been identified (Albarracin et al., 1994; Fan et al., 1995; Cam-

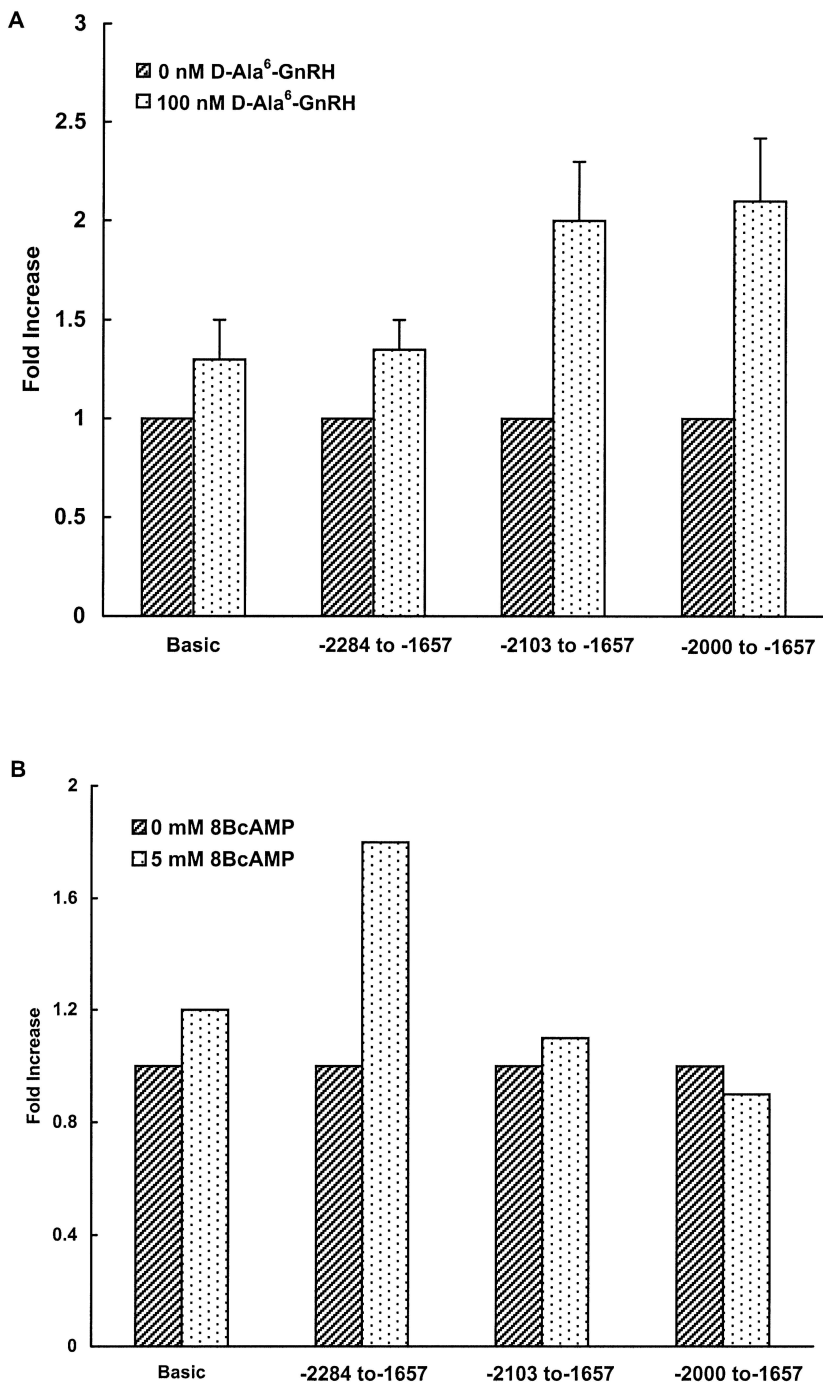


Fig. 7. Effect of 8-bromo cAMP and GnRH agonist (D-Ala⁶-GnRH) on GnRH receptor promoter activity. The luciferase chimeric constructs containing the various 5' flanking sequence of the human GnRH receptor gene were co-transfected with pSV- β -gal vector into α T31 cells. After 24 h of transfection, the cells were treated with either 8-bromo cAMP (A) or GnRH agonist (B). After 24 h of treatment the cells were lysed and assayed for luciferase and galactosidase activities. Luciferase activity was normalized with galactosidase activity. The results presented are the mean of three independent experiments.

pion et al., 1996; Duval et al., 1997b; Kakar, 1997; Pincas et al., 1998). Using the 5' flanking region of the human GnRH receptor gene sequence and its 5' and 3' deletion constructs, Ngan et al. (1999) reported the importance of the most proximal 173 bp region (relative to translation start site) within the first exon, which is not a promoter itself but instead contains a critical regulatory element(s) essential for the basal expression of the human GnRH receptor gene. These investigators also reported the existence of three putative gonadotrope-specific elements (GSE; consensus 5'-CTGA/TCCTTG-3') residing at positions -5, -134 and -396. Using the 2300 bp of the flanking region of the human GnRH receptor gene, Cheng et al. (2000a,b) showed a dose-dependent and time-dependent down-regulation of GnRH receptor promoter activity, suggesting the regulation of GnRH receptor gene by GnRH and its analog. By performing the progressive 5' deletion studies, these investigators also identified a 248-bp DNA fragment (-1018 to -771), relative to the translation start site, that appears to be responsible for the GnRH analog-mediated down-regulation of the human GnRH receptor gene (Cheng et al., 2000a,b). Two AP1 binding sequences also were also identified within the 248 bp sequence, and mutation of these was shown to abolish the GnRH analog-induced response. In contrast, Norwitz et al. (1999) and White et al. (1999) reported stimulation of the mouse GnRH receptor promoter by 100 nM of GnRH agonist (GnRHa). Duval et al. (1997b), using mutation analysis of the 600 bp 5' flanking region, that has been shown to mediate basal expression of the mouse receptor gene, located a GnRH-responsive fragment, in which a putative AP-1 was subsequently identified (White et al., 1999). This GnRHa-induced increase in promoter activity was mimicked by treatment with phorbol ester, but not by forskolin. Furthermore, pretreatment of the cells with a specific protein kinase C inhibitor (GF109203X) blocked the GnRHa- and TPA-induced increase in promoter activity, suggesting an important role for PKC in regulation of the expression of the mouse GnRH receptor gene. Norwitz et al. (1999), by deleting the 5' and 3' sequence of 1.2 kb flanking region of the mouse GnRH receptor gene sequence, identified a 68 bp region located between -300 and -232 (relative to the ATG site) that is responsible for the GnRHa-induced

stimulation. Furthermore, mutation of the AP-1 binding sequence resulted in complete loss of promoter activity, suggesting the importance of the AP-1 binding sequence in GnRH responsiveness. Consistent with these results concerning the mouse GnRH receptor gene, our analysis of a 346 bp sequence in the flanking region of the human GnRH receptor gene (-2003 to -1657) showed activation of promoter activity by GnRH in α T31 cells (Fig. 7A).

In the mouse, the 500 bp sequence upstream of the ATG site was found to be sufficient to drive the promoter activity in α T31 cells. Deletion and mutation analysis of this region allowed the identification of a number of regulatory elements including SF-1 and AP-1 as well as a novel element referred to as the GnRH receptor activating sequence (GRAS: 5'-CTAGTCACAACA-3'). Each of these elements appears to be important in the regulation of expression of the receptor gene as their mutation resulted in a 60% to 80% reduction in promoter activity (Clay et al., 1995; Duval et al., 1997a). In the rat, a 1.2 kb flanking sequence was found to be sufficient to drive the basal promoter activity. However, in this species, various regulatory elements including LH-2 (-869 to -862, relative to ATG site), and SF-1 (-696 to -688, relative to ATG site) were found to be responsible for the regulation of the promoter activity, as mutation of these sequences resulted in a decrease in promoter activity (Duval et al., 1997b).

cAMP may also have a role in regulating GnRH receptor gene expression. The existence of a CRE (cAMP response element) in the flanking regions of the rat, mouse and human GnRH receptor genes has been reported (Albarracin et al., 1994; Fan et al., 1995; Kakar, 1997; Reinhart et al., 1997). To determine if the CRE in the human receptor gene is functional, we tested the 5' flanking region (-2284 to -1657, containing CRE sequence) of the gene in α T31 cells after its subcloning into the pGL3 vector. As shown in Fig. 7B, treatment of the transfected α T31 cells with the cAMP analog (8-bromo cAMP) resulted in a two-fold increase in luciferase activity. Deletion of the CRE sequence (-2284 to -2104) abolished the cAMP analog effect. In addition, forskolin, an adenylate cyclase activator, mimicked the effect of the cAMP analog (results not shown), suggesting that the CRE sequence in human GnRH receptor gene is functionally active. Similar

results were reported by Lin and Conn (1998), Maya-Nunez and Conn (1999) and Maya-Nunez and Conn (2001) for the mouse GnRH receptor gene. These investigators localized the CRE sequence at -107 to -100 (relative to ATG start site) in the mouse gene.

Hormonal regulation of GnRH receptor gene expression

It is well documented that pituitary GnRH receptor is regulated by GnRH. Radioreceptor assays revealed that castration increases GnRH receptor binding, and that testosterone in the male, and estradiol and progesterone in the female prevent this rise (Clayton and Catt, 1981a,b). These effects are likely due to gonadal steroid suppression of GnRH secretion since a GnRH antiserum blocked the castration-associated rise in GnRH receptors (Frager et al., 1981). By contrast, in the mouse pituitary, GnRH receptor concentration was decreased by orchidectomy to 45% below intact values for up to 3 months even though there is a persistent 5-fold rise in serum LH values. Further, treatment of male mice with testosterone propionate prevented the GnRH receptor fall (Naik et al., 1994). A seminal finding was that GnRH up-regulates its receptor only when delivered to gonadotropes in a pulsatile fashion. On the other hand, continuous GnRH causes receptor down-regulation and desensitization. This finding in part led to the development of long-acting GnRH analogs that produce a chemical gonadectomy and are widely used to treat patients with prostate cancer, precocious puberty, and many other disorders.

Much of the regulation of GnRH receptor protein is through effects on GnRH receptor gene expression. The level of GnRH receptor mRNA varies during the rat estrus cycle, and increases following orchidectomy or ovariectomy in a wide variety of species (Kaiser et al., 1993; Kakar et al., 1994a) including primates (Winters et al., 2001). Experiments using perfused rat pituitary cells indicated that only pulsatile GnRH increases the level of GnRH receptor mRNA (Kaiser et al., 1993), but how this occurs remains poorly understood.

Although estradiol reduces GnRH receptor *in vivo* in rats (Kaiser et al., 1993; Kakar et al., 1994a), estradiol increases GnRH-R mRNA levels dose-dependently in male and female sheep (Turzillo

et al., 1994; Adams et al., 1996). This effect is likely to be directly on the pituitary since it can be mimicked in ovine pituitary cell cultures (Ghosh et al., 1996). Progesterone decreases GnRH binding sites and GnRH receptor mRNA concentrations in ovine pituitary cells (Laws et al., 1990; Sealfon et al., 1990). In primary cultures of female rat pituitary cells, however, neither estradiol nor progesterone modulates the basal expression of GnRH receptor mRNA (Cheon et al., 2000). Kakar et al. (1993) found increased levels of GnRH receptor mRNA in sterile cows. In mouse-derived α T31 cells, estradiol reduces GnRH receptor number as well as the efficiency of coupling of residual GnRH receptors to second messenger generation (McArdle et al., 1992). Approximately 9,100 bp of 5' flanking region from the ovine GnRH receptor (oGnRHR) gene was devoid of transcriptional activity and unresponsive to both estradiol and GnRH in α T31 cells whereas this same 9,100 bp promoter fragment directed tissue-specific expression of luciferase in multiple lines of transgenic mice (Duval et al., 2000). The absence of an estrogen response element (ERE) in the flanking region of the (human) GnRH receptor gene suggests that regulation by estrogen may not be transcriptional.

Using the human GnRH receptor 5' flanking sequence, Cheng et al. (2001) found inhibition of promoter activity by progesterone in α T3-1 cells, but activation in placental JEG-3 cells. Using 5' and 3' deletion analysis, these investigators localized the progesterone response element (PRE) between -535 to -531 (relative to ATG). Furthermore the physiological effects of progesterone were shown to occur through two different isoforms of the progesterone receptor. Over-expression of PR-A reduced promoter activity in both pituitary and placental cells, whereas over-expression of PR-B activated the promoter in placental cells but inhibited the promoter in pituitary cells.

An additional autocrine/paracrine mechanism for GnRH receptor mRNA regulation is suggested by the findings that rat GnRH receptors are up-regulated by activin (Braden and Conn, 1992), and decreased by inhibin (Wang et al., 1989) in rat pituitary cell cultures. Activin stimulated transcription of GnRH receptor mRNA in α T3-1 cells (Fernandez-Vazquez et al., 1996), and inhibin suppressed GnRH receptor

steady state mRNA levels in male rats (Winters et al., 1996). On the other hand, inhibin increases GnRH receptor mRNA concentrations in ovine pituitary cultures (Sealfon et al., 1990).

Finally, transcription of the GnRH receptor gene is stimulated by PACAP presumably through a cAMP pathway (Pincas et al., 2001). The region between -1727 and -1577 was found to be responsible for PACAP induction (Ngan et al., 2001). This finding expands the interplay between these two hypophysiotropic peptides.

Summary

In summary, isolation of GnRH receptor cDNA, its gene, and identification of regulatory elements in the flanking region of the gene have added to our knowledge regarding the tissue-specific expression of the GnRH receptor gene, and the mechanisms that mediate and influence its transcriptional regulation. However, the interactions of the different regulatory factors (nuclear factors) and the effects of these interactions on the regulation of the GnRH receptor gene remain unclear. Due to existence of multiple promoters and transcriptional start sites in human GnRH receptor gene and the lack of a human gonadotrope cell line, the precise promoter and transcriptional start sites in human pituitary, extra-pituitary tissues and tumors have not yet been identified.

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SECTION III

Physiology and regulation

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Hypothalamic control of gonadotropin secretion[‡]

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Introduction

The control of gonadotropin secretion is extremely complex as revealed by the research of the past 40 years since the discovery of LHRH (McCann et al., 1960), now commonly called gonadotropin-releasing hormone (GnRH) (McCann and Ojeda, 1996). This was the second of the hypothalamic-releasing hormones characterized. It stimulates FSH release, albeit in smaller amounts than LH. For this reason, it was renamed GnRH (Reichlin, 1992; McCann and Ojeda, 1996). Overwhelming evidence indicates that there must be a separate FSHRH because pulsatile release of LH and FSH can be dissociated. In the castrate male rat, roughly half of the FSH pulses occur in the absence of LH pulses and only a small fraction of the pulses of both gonadotropins are co-incident. LHRH antisera or antagonists can suppress

pulsatile release of LH without altering FSH pulses (Culler and Negro-Vilar, 1987; McCann et al., 1993). LH, but not FSH pulses can be suppressed by alcohol (Dees et al., 1985), delta-9-tetrahydrocannabinol, and cytokines, such as interleukin-1 alpha (IL-1 α) (Rettori et al., 1991). In addition, a number of peptides inhibit LH, but not FSH release and a few stimulate FSH without affecting LH (McCann and Krulich, 1989; McCann et al., 1993). The hypothalamic areas controlling LH and FSH are separable. Stimulation in the dorsal medial anterior hypothalamic area causes selective FSH release, whereas lesions in this area selectively suppress the pulses of FSH and not LH (Lumpkin et al., 1989). Conversely, stimulations or lesions in the medial preoptic region can augment or suppress LH release respectively without affecting FSH release. Electrical stimulation in the preoptic region releases only LH, whereas lesions in this area inhibit LH release without inhibiting FSH release. The medial preoptic area contains most of the perikarya of LHRH neurons. The axons of these neurons project from the preoptic region to the anterior and mid portions of the median eminence. Extracts of the anterior-mid median eminence contain LH-releasing activity commensurate with the content of immunoassayable LHRH, whereas extracts of the caudal median eminence

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[‡] Modified from *Archives of Medical Research*, 32: 476–485, 2001.

and organum vasculosum lamina terminalis (OVLT) contain more FSH-releasing activity than can be accounted for by the content of LHRH (McCann et al., 1993). Lesions confined to the rostral and mid-median eminence can selectively inhibit pulsatile LH release without altering FSH pulsations, whereas lesions that destroy the caudal and mid-median eminence can selectively block FSH pulses in castrated male rats (McCann et al., 1993; Marubayashi et al., 1999). Therefore, the putative FSHRH may be synthesized in neurons with perikarya in the dorsal anterior hypothalamic area with axons that project to the mid and caudal median eminence to control FSH release selectively.

FSHRH

We (followed by several other groups) reported FSH-releasing activity in the stalk-median eminence (Igarashi and McCann, 1964). The activity was purified and separated from the LH-releasing activity in 1965 as measured by *in vivo* bioassays (Dhariwal et al., 1965, 1967). This separation was confirmed by Schally et al. (1966) and two other laboratories. It has repeatedly been shown that bioactive and radioimmunoassayable FSH-releasing activity can be separated from LHRH by gel filtration through sephadex G-25 on the same column used in the earlier research. FSH- and LH-releasing activity were assayed by the increase in plasma FSH and LH, respectively, in ovariectomized, estrogen–progesterone blocked rats (Lumpkin et al., 1987). The separation of the two activities was also demonstrable by assay of FSH and LH released from hemipituitaries incubated *in vitro* by bioassay (Mizunuma et al., 1983) and RIA (Yu et al., 2000). In both assay systems, FSHRH emerged from the column just before elution of LHRH.

In the search for FSHRH, we first believed that it might be an analog of LHRH (Dhariwal et al., 1967) and later had many such analogs synthesized. We tested the forms of GnRH that were known to exist in lower species (Yu et al., 1990, 2002). We had not tested lamprey GnRH-III, but when we realized that an antiserum that crossed reacted with IGnRH-I and IGnRH-III, immunostained neural fibers in the arcuate nucleus proceeding to the median eminence of human brains obtained at autopsy, it occurred to us

that IGnRH-III could be the FSHRH since IGnRH-I had little activity to release either LH or FSH (Yu et al., 1997a). Indeed, IGnRH-III is a potent FSH-releasing factor with little or no LH-releasing activity both *in vitro* when incubated with hemipituitaries of male rats and *in vivo* when injected into conscious, ovariectomized, estrogen–progesterone-blocked rats (Yu et al., 1997a). The lowest dose tested in that preparation (10 pM) produced a highly significant increase in plasma FSH with no increase in LH, a 10-fold higher dose (100 pM) increased plasma FSH similarly and had no effect on LH release. IGnRH-III 10^{-9} M produced highly significant FSH release *in vitro*, whereas, LH-releasing activity did not appear until 10^{-6} M (Yu et al., 1997a).

Testing other GnRH analogs in this *in vitro* system revealed that cGnRH-II had no significant activity to release FSH or LH until a much higher dose was reached and no selective releasing activity. It had slightly selective FSH-releasing activity *in vivo* in ovariectomized, estrogen–progesterone-blocked rats (Yu et al., 1990). We have tested over 40 natural and synthesized analogs of IGnRH-III and found that the amino acids in positions 5, 6, 7 and 8 of IGnRH-III are important in conveying selective FSH-releasing activity. These 4 amino acids are dissimilar from those in LHRH. From tunicate to man the first 4 amino acids in GnRHs are nearly constant and the last 2 positions, 9 and 10, are constant, leading us to conclude that amino acids 5–8 are crucial for determining whether or not an analog is going to be selective for FSH or LH release (Yu et al., 1999, 2002). These 4 amino acids in IGnRH-III are His₅, Asp₆, Trp₇ and Lys₈; whereas in mLHRH they are Tyr₅, Gly₆, Leu₇, and Arg₈. Thus, IGnRH III has 60% homology with LHRH.

We fractionated 1,000 rat hypothalami by gel filtration on Sephadex G-25 and determined the FSH- and LH-releasing activity of the various fractions by bioassay on male rat hemipituitaries and compared the localization of these activities with that of LHRH and IGnRH determined by RIA. IGnRH was assayed by RIA using a specific antiserum for IGnRH, that recognized IGnRH I and III equally but did not cross-react with LHRH or cGnRH-II. A peak of LHRH immunoreactivity was found as well as 3 peaks of IGnRH immunoreactivity that preceded the peak of LHRH. The first peak eluted was quite small.

The second peak was much larger and the third peak was of greatest magnitude. Only the second peak altered gonadotropin release and it produced selective FSH release.

To determine whether this activity was caused by IGnRH, anterior hemipituitaries were incubated with normal rabbit serum or the IGnRH antiserum (1 : 1,000) and the effect on FSH- and LH-releasing activity of the FSH-releasing fraction and the LH-releasing activity of LHRH was determined. The antiserum had no effect on basal release of either FSH or LH, but eliminated the FSH-releasing activity of the active fraction without altering the LH-releasing activity of LHRH. Since IGnRH-I has minimal non-selective activity to release FSH or LH, whereas, previous experiments had shown that IGnRH-III highly selectively releases FSH with a potency equal to that of LHRH to release LH, these results support the hypothesis that the FSH-releasing activity observed in the FSHRH fraction was caused by IGnRH-III or a very closely related peptide (Yu et al., 2000).

Localization of IGnRH in the brain of rats by immunocytochemistry

Using the same antiserum that we employed to find the location of IGnRH after gel-filtration of rat hypothalami, we attempted to localize IGnRH neurons by immunocytochemistry. Immunoreactive IGnRH-like cell bodies were found in the ventromedial preoptic area with axons projecting to the rostral wall of the third ventricle (3V) and OVLT. Another population of IGnRH-like cell bodies was located in the dorsomedial preoptic area with axons projecting caudally and ventrally to the external layer of the median eminence. On the other hand, using a highly specific, monoclonal antiserum against mGnRH to localize the mGnRH neurons, so that their localization could be compared with that of IGnRH neurons, we found that there were no mGnRH cells or fibers in the dorsomedial preoptic area that contained perikarya and fibers of IGnRH neurons (Dees et al., 1999).

Furthermore, immunoabsorption studies indicated that the cell bodies of the IGnRH neurons were eliminated by IGnRH-III, but not by mGnRH whereas the axons in the median eminence were eliminated by IGnRH-III but only slightly reduced by absorption with mGnRH. Using an antiserum against cGnRH-II

that visualized cGnRH-II neurons in the chicken hypothalamus, no such neurons could be visualized in the rat hypothalamus (Dees et al., 1999).

Since the IGnRH antiserum (#3952) recognized IGnRH-I and IGnRH-III equally, a specific antiserum against IGnRH-III without cross-reactivity with IGnRH-I was needed to prove that the IGnRH neurons visualized in the rat brain were indeed IGnRH-III neurons. Our recent studies indicate that the specific IGnRH-III antiserum (#39-82-78-3) visualizes the same population of neurons seen with the less specific IGnRH antiserum (3952). Also, the staining of cells and fibers could be eliminated by IGnRH-III but was not affected by IGnRH-I or mGnRH. Consequently, the results strongly support the hypothesis that the IGnRH-III neurons are located in the areas of the brain responsible for control of FSH are the FSHRH neurons (Hiney et al., 2002). The IGnRH-III neurons whose cell bodies are located in the caudal dorsal medial preoptic area with axons projecting to the median eminence are in the very regions which have been shown to be selectively involved in the control of FSH release by lesion and stimulation studies described earlier (pages 151 and 152). Lesions in the caudal dorsal medial preoptic and anterior dorsal medial anterior hypothalamic area blocked the castration-induced rise in FSH (Lumpkin and McCann, 1984) and blocked pulsatile FSH release without interfering with pulsatile LH release (Lumpkin et al., 1989). Conversely, implants of prostaglandin E₂ (PGE₂) in this region evoked selective FSH release. These evoked a pattern of FSH selective release from implants of PGE₂ along a path running from the medial dorsal preoptic area to the caudal parts of the median eminence regions (Ojeda et al., 1977) that have been shown to contain more FSH-releasing activity than could be accounted for by LHRH, and that when destroyed can also selectively block FSH and not LH pulses in castrate male rats (Marubayashi et al., 1999).

The other population of IGnRH-III neurons in the ventromedial caudal preoptic area has axons that appear to project to the OVLT and to the wall of the 3V, whereas the more dorsal neurons also project to the ventricle and caudally to the mid-brain central gray along the same pathway as the LHRH neurons (Dees et al., 1999). The projection to the mid-brain central gray suggests the possibility that IGnRH-III may be

involved in mating behavior since this is the area which LHRH activates to induce mating behavior.

The function of the caudal ventral medial preoptic neurons projecting to the OVLT is not clear. Possibly, they release lGnRH-III into the ventricular system for actions more caudally after its uptake from the ventricle. Alternatively, their axons may in some manner also reach the median eminence by an unknown trajectory. It is interesting that some of these neurons in this region also contain mLHRH and that there are other neurons there that contain only LHRH. These are located laterally to the positions of the lGnRH-III neurons that are more medial. The fact that some of the neurons contain both peptides suggests that they may interact in an ultra short-loop feedback mechanism to control the release not only of FSHRH, but also LHRH (Hiney et al., 2002). The localization and projection of the lGnRH-III and LHRH neurons are illustrated (Fig. 1).

Evidence for a specific FSHRH receptor

The selectivity of release of FSH by FSHRH and lGnRH-III suggests the probability of a specific FSHRH receptor on pituitary gonadotropes. In an early experiment in collaboration with J. Rivier and Vale (Salk Institute, La Jolla, CA), we tested the FSH-releasing activity of our purified FSHRH using monolayered cultured pituitary cells and were unable to detect any FSH-releasing activity (unpublished data). This led us to the hypothesis that the FSHRH receptors are down regulated during the 4 days in culture in the absence of gonadal steroids. Indeed, in a recent experiment, we have confirmed the fact that lGnRH-III has little activity on monolayered cultured male rat pituitary cells at the same time that LHRH is active at 10^{-10} M. lGnRH-III was also tested in a LHRH receptor assay. The production of inositol phosphate in COS cells transfected with LHRH receptors was not increased by lGnRH-III until a concentration of 10^{-7} M. As the concentration was increased full activity was obtained at 10^{-4} M (Yu et al., 1999, 2002). We hypothesized that we might demonstrate the presence of lGnRH-III receptors on gonadotropes using biotinylated lGnRH-III and mLHRH. To enhance imaging an avidin-based system was used combined with immunocytochemistry for FSH and LH. The lGnRH-III

peptide was extended with one or two spacer arms of aminocaproic acid between the biotin moiety and one amino acid of the peptide. Three biotinylated derivatives of lGnRH-III were tested for bioactivity. The peptide derivative biotinylated on Lys⁸ with 2 spacer arms of aminocaproic acid (Bxx-Lys⁸-lGnRH-III) had selective FSH-releasing activity at a dose of 10^{-8} M on hemipituitaries incubated in vitro, whereas the analog with one spacer arm Bx-Lys⁸) had less activity than that of the above derivative. By contrast, the analog biotinylated on Asp⁶ and one spacer arm (Bx-Asp⁶) had no selective FSH-releasing activity. Therefore, the two Bxx-lGnRH-III appeared to be satisfactory for locating lGnRH-III binding sites on pituitary gonadotropes. Biotinylated lGnRH-III (10^{-9} M) bound to 80% of FSH gonadotropes and only 50% of LH gonadotropes of acutely dispersed pituitary cells, a finding that indicates that there are receptors on gonadotropes that bind this peptide (Childs et al., 2001). The binding of biotinylated lGnRH-III was not displaced with LHRH that indicates that it is highly specific for the putative FSHRH receptors. It appears that in this situation as with monolayer cultured pituitary cells, the FSHRH receptors disappear with time in culture since with 24 h in culture, the binding of the biotinylated lGnRH-III was significantly decreased. We believe on the basis of our recent studies with the biotinylated lGnRH-III that ultimately a specific receptor for this peptide will be found in the pituitary and we believe it is in all probability the FSHRH.

lGnRH-III binds to the 3 GnRH receptors discovered in the bullfrog, but the affinity to the receptors was less than that of mLHRH or cGnRH-II as determined in the inositol phosphate assay (Wang et al., 2001). Neill et al. (2001) have recently reported the existence of a second GnRH receptor (GnRH-IIr) in the human genome and reported the cloning and characterization of its cDNA from monkeys. The cDNA generated a G protein coupled transmembrane receptor having a C-terminal cytoplasmic tail, whereas GnRH-Ir lacks this tail. GnRH-IIr resembles more closely the type II receptors of amphibians and fish than it does the type I receptor of humans. This receptor is specific for cGnRH-II and is expressed ubiquitously in human tissues (Neill et al., 2001).

Previously, cGnRH-II has been reported in various tissues in monkeys and humans, but the peptide

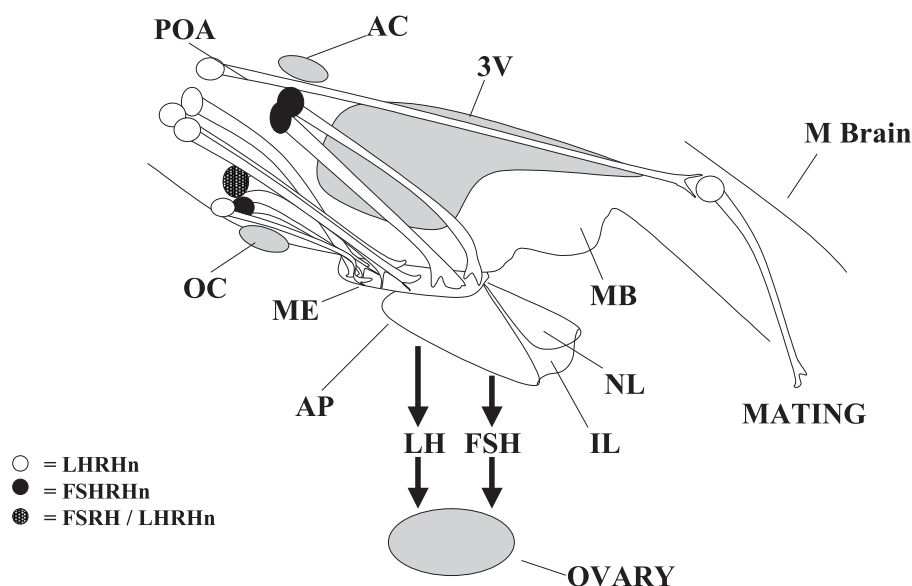


Fig. 1. Parasagittal section of preoptic and hypothalamic region of the rat brain illustrating the distribution of FSHRH and LHRH neurons. Abbreviations: POA = preoptic area, AC = anterior commissure, OC = optic chiasm, 3V = third ventricle, ME = median eminence, MB = mammillary body, M Brain = midbrain, AP = anterior pituitary, IL = intermediate lobe, NL = neural lobe.

was found in the hypothalamus only of fetal monkeys and also in the mid-brain central gray (Lescheid et al., 1997). In adult monkeys, there were only a few cells and fibers in the caudal hypothalamus suggesting that this peptide would not reach the pituitary via the portal vessels, but must have other actions perhaps on mating behavior or in regulation of cell division. Indeed, as indicated above, we found no cGnRH-II perikarya and only scant fibers in the hypothalamus of the rat in contrast with the readily observed lGnRH-III neurons and terminals in the median eminence (Hiney et al., 2002). Furthermore, cGnRH-II, as indicated above, has little or no selective FSH releasing activity; however, there is little doubt that it is an ancient GnRH existing from fish to mammals.

Mechanism of action of FSHRH, LHRH and leptin on gonadotropin secretion

It is well known that FSH and LH release is controlled by calcium ions (Ca^{2+}) (Wakabayashi et al., 1969; Stojilkovic, 1998) and that interaction of LHRH with its receptor causes an increase in intracellular free calcium and also activates the phos-

phatidyl inositol cycle that mobilizes internal calcium. The resulting increase in intracellular free calcium mediates the releasing action of LHRH (for review, see Stojilkovic, 1998); however, we earlier showed a role for a cGMP and not cyclic adenosine monophosphate (cAMP) in controlling the release of LH and FSH mediated by LHRH (Nakano et al., 1978; Naor et al., 1978; Snyder et al., 1980). This was before it was accepted that NO is a physiologically significant, gaseous transmitter that acts by activation of guanylyl cyclase that converts GTP to cGMP. cGMP activates protein kinase G that causes exocytosis of gonadotropin secretory granules.

To test the hypothesis that the FSH-releasing activity of lGnRH-III (or FSHRH) is mediated by calcium and NO, calcium was removed from the medium and a chelating agent (ethylene glycol-N-N'-N'-tetraacetic acid) that would remove any residual Ca^{2+} was added. The action of purified FSHRH and lGnRH was blocked in the absence of Ca^{2+} . N^G -monomethyl-L-arginine (NMMA), a competitive inhibitor of NOS, was added to the medium in other experiments. We found that this competitive inhibitor of NOS, NMMA, completely blocked the FSH-releasing activity of not only purified FSHRH

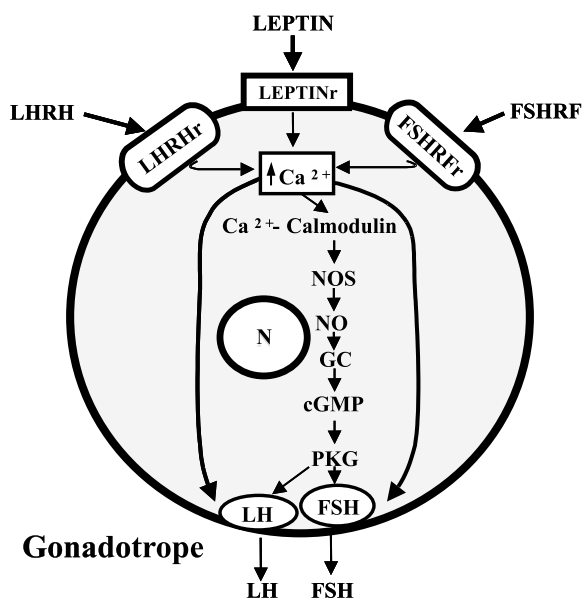


Fig. 2. Schematic diagram illustrating the mechanism of the gonadotropin-releasing action of FSHRH, LHRH and leptin, N = nucleus, other abbreviations are in list of abbreviations. The principal pathway is via NO, cGMP and PKG, but Ca^{2+} may have an independent action.

but also of IGnRH-III. Furthermore, sodium nitroprusside (NP), a releaser of NO, stimulated both LH and FSH release and the activity of LHRH to release both LH and FSH was also blocked by NMMA. These data indicate that FSHRH (or IGnRH-III) acts on its putative receptor via a calcium-dependent, nitric oxide pathway to release FSH specifically, whereas LHRH acts on its receptor similarly to increase intracellular Ca^{2+} that activates NOS in the gonadotrophs to cause release of LH and to a lesser extent FSH (Yu et al., 1997c, 1999, 2002) (Fig. 2).

Possible use of IGnRH-III in control of reproduction

Not only does IGnRH-III have selective FSH-releasing activity in vitro but also in vivo in the ovariectomized, estrogen–progesterone-blocked rat (Yu et al., 1997a) and normal male rat (Yu et al., unpublished), but also in the cow (Dees et al., 2001). Treatment of cows with IGnRH-III in the luteal stage of the estrous cycle has produced multiple large follicles in 6 consecutive treatment cycles, in striking

contrast to the normal development of a single large follicle in each cycle. Ovulation of 2 large follicles occurred in 2 cases following injection of human chorionic gonadotropin. On the basis of these findings we believe that IGnRH-III has great potential to alter reproduction in animals and man (Dees et al., 2001).

Role of NO in control of LHRH release

NO is formed in the body by NOS, an enzyme which converts arginine in the presence of oxygen and several cofactors into equimolar quantities of citrulline and NO. There are three isoforms of the enzyme. One of these, nNOS, is found in the cerebellum and various regions of the cerebral cortex and also in various ganglion cells of the autonomic nervous system. Large numbers of nNOS-containing neurons, termed NOergic neurons, were also found in the hypothalamus particularly in the paraventricular and supraoptic nuclei with axons projecting to the median eminence and neural lobe, which also contains large amounts of nNOS. These findings indicated that the enzyme is synthesized at all levels of the neuron from perikarya to axon terminals (McCann and Rettori, 1997).

Because of this distribution in the hypothalamus in regions that contain peptidergic neurons that control pituitary hormone secretion, we decided to determine the role of this soluble gas in the release of LHRH. The approach used was to use sodium NP that spontaneously liberates NO to see if this altered the release of various hypothalamic transmitters. Hemoglobin, which scavenges NO by a reaction with the heme group in the molecule and inhibitors of NOS, such as NMMA, a competitive inhibitor of NOS, were used to determine the effects of decreased NO. Two types of studies were performed. In the first set of experiments, medial basal hypothalamic (MBH) explants were preincubated in vitro and then exposed to neurotransmitters that modify the release of various hypothalamic peptides in the presence or absence of inhibitors of the release of NO. The response to NO itself, provided by sodium NP, was also evaluated. To determine if the results in vitro also held in vivo, substances were microinjected into the 3V of the brain of conscious, freely moving animals to determine the effect on pituitary hormone release (Rettori et al., 1993).

Our most extensive studies were carried out with regard to the release of LHRH. Not only does LHRH act after its secretion into the hypophyseal portal vessels to stimulate LH and to a lesser extent FSH release, but it also induces mating behavior in female rats and penile erection in male rats by hypothalamic action (Mani et al., 1994).

Our experiments showed that release of NO from sodium NP *in vitro* promoted LHRH release and that the action was blocked by hemoglobin, a scavenger of NO. NP also caused an increased release of PGE₂ from the tissue, which previous experiments showed played an important role in release of LHRH. Furthermore, it caused the biosynthesis and release of prostanoids from ¹⁴C arachidonic acid. The effect was most pronounced for PGE₂, but there also was release of lipoxygenase products that have been shown to play a role in LHRH release. Inhibitors of cyclooxygenase, the responsible enzyme for prostanoid synthesis, such as indomethacin and salicylic acid blocked the release of LHRH induced by norepinephrine (NE), providing further evidence for the role of NO in the control of LHRH release via the activation of cyclooxygenase-1. Nedleman's group later showed that NO activates cyclooxygenase-1 and cyclooxygenase-2 in cultured fibroblasts. The action is probably mediated by combination of NO with the heme group of cyclooxygenase altering its conformation. The action on lipoxygenase is similar; although it contains ferrous iron, the actual presence of heme in lipoxygenase has yet to be demonstrated (Rettori et al., 1992, 1993).

As indicated above (page 155), the previously accepted pathway for the physiologic action of NO is by activation of soluble guanylate cyclase by interaction of NO with the heme group of this enzyme, thereby causing conversion of GTP into cGMP, which mediates the effect on smooth muscle by decreasing the intracellular [Ca²⁺]. On the other hand, Muellam's group showed in incubated pancreatic acinar cells that cGMP has opposite effects on intracellular [Ca²⁺], elevating it at low concentration and lowering it at higher concentrations. We postulate that the NO released from the NOergic neurons, near the LHRH neuronal terminals, increases the intracellular free calcium required to activate phospholipase A₂ (PLA₂). PLA₂ causes the conversion of membrane phospholipids in the LH terminal to arachido-

nate, which then can be converted to PGE₂ via the activated cyclooxygenase. The released PGE₂ activates adenylyl cyclase causing an increase in cAMP release, which activates protein kinase-A, leading to exocytosis of LHRH secretory granules into the hypophyseal portal capillaries for transmission to the anterior pituitary gland (Canteros et al., 1995).

NE has previously been shown to be a releaser of LHRH. It acts by activation of the NOergic neurons because the activation of these neurons and the release of LHRH could be blocked by a competitive inhibitor of NOS, NMMA. NE acts to stimulate the release of NO from the NOergic neurons by α_1 adrenergic receptors since its action can be blocked by phentolamine, an α receptor blocker, and prazosine, a specific α_1 receptor blocker. Activation of the α_1 receptors is postulated to increase intracellular [Ca²⁺] that combines with calmodulin to activate NOS leading to generation of NO.

We measured the effect of NE on the content of NOS in the MBH explants at the end of the experiments by homogenizing the tissue and adding ¹⁴C arginine and measuring its conversion to citrulline on incubation of the homogenate. Because arginine is converted to equimolar quantities of NO and citrulline, measurement of citrulline production provides a convenient estimate of the activity of the enzyme. The NO disappears rapidly making its measurement very difficult. NE caused an increase in the apparent content of the enzyme. That we were actually measuring enzyme content was confirmed, because incubation of the homogenate with L-nitroarginine methyl ester, another inhibitor of NOS caused a drastic decline in the conversion of arginine to citrulline. We further confirmed that we actually had increased the content of enzyme by isolating the enzyme according to the method Bredt and Snyder (Rettori et al., 1992) and then measuring the conversion of labeled arginine to citrulline. The conversion was highly significantly increased by NE (Canteros et al., 1996).

Glutamic acid (GA), at least in part by n-methyl-D-aspartate receptors, also plays a physiologically significant role in controlling the release of LHRH. Therefore, we evaluated where GA fits into the picture. It also acted via NO to stimulate LHRH release, but we showed that the effect of GA could be completely obliterated by the α -receptor blocker phentolamine. Consequently, we concluded that GA

acted by stimulation of the noradrenergic terminals in the MBH to release NE, which then initiated NO release and stimulation of LHRH release (Kamat et al., 1995).

Oxytocin has actions within the brain to promote mating behavior in the female and penile erection in the male rat. Since LHRH mediates mating behavior, we hypothesized that oxytocin would stimulate the LHRH release that, after secretion into the hypophyseal portal vessels, mediates LH release from the pituitary. Consequently, we incubated MBH explants and demonstrated that oxytocin (10^{-7} – 10^{-10} M) induced LHRH release via NE stimulation of nNOS. Therefore, oxytocin may be very important as a stimulator of LHRH release. Furthermore, NO acted as a negative feedback to block oxytocin release (Rettori et al., 1997).

One of the few receptors to be identified on LHRH neurons is the gamma amino butyric acid (GABA_A) receptor. Consequently, we evaluated the role of GABA in LHRH release and the participation of NO in this. The experiments showed that GABA blocked the response of the LHRH neurons to NP that acts directly on the LHRH terminals. We concluded that GABA suppressed LHRH release by blocking response of the LHRH neuronal axons to NO. Additional experiments showed that NO stimulates the release of GABA, providing thereby an inhibitory feed-forward pathway to inhibit the pulsatile release of LHRH initiated by NE. As NE stimulated the release of NO, this would stimulate the release of GABA, which would then block the response of the LHRH neuron to the NO released by NE (Seilicovich et al., 1995a).

Other studies indicated that NO would suppress the release of dopamine and NE. We have already described the ability of NE to stimulate LHRH release and dopamine also acts as a stimulatory transmitter in the pathway. Therefore, there is an ultra short-loop negative feedback mechanism to terminate the pulsatile release of LHRH because the NO released by NE would diffuse to the noradrenergic terminals and inhibit the release of NE, thereby terminating the pulse of NE, LHRH and finally LH (Seilicovich et al., 1995b).

We further examined the possibility that other products from this system might have inhibitory actions. Indeed, we found that as we added increasing

amounts of NP, we obtained a bell-shaped dose-response curve of the release of LHRH, such that the release increased with increasing concentrations of NP up to a maximum at around 600 μ M and then declined with higher concentrations. When the effect of NP on NOS content at the end of the experiment was measured, we found that high concentrations of NP lowered the NOS content. Furthermore, NP could directly decrease NOS content when incubated with MBH homogenates, results that indicate a direct effect on NOS probably by interaction of NO with the heme group on the enzyme. Thus, when large quantities of NO are released, as could occur after the induction of inducible (i)NOS in the brain during infections, the release of NO would decrease by an inhibitory action on the enzyme at these high concentrations. Furthermore, high concentrations of cGMP released by NO also acted in the explants or even in the homogenates to suppress the activation of NOS. This pathway could also be active in the presence of high concentrations of NO, such as would occur in infection by induction of iNOS by bacterial or viral products (Canteros et al., 1996).

The effect of cytokines (IL-1 and granulocyte macrophage colony-stimulating factor (GMCSF)) on the NOergic control of LHRH release

The cytokines so far tested, for example IL-1 and GMCSF, act within the hypothalamus to suppress the release of LHRH as revealed in both in vivo and in vitro studies. We have examined the mechanism of this effect and found that for IL-1, it occurs by inhibition of cyclooxygenase as shown by the fact that there is blockage of the conversion of labeled arachidonate to prostanoids, particularly PGE₂, and the release of PGE₂ induced by NE is also blocked (Rettori et al., 1994).

A principle mechanism of action is by suppression of the LHRH release induced by NO donors such as NP. We first believed that there were IL-1 and GMCSF receptors on the LHRH neuron that blocked the response of the neuron to NO. However, because we had also shown that GABA blocks the response to NP and earlier work had shown that GABA receptors are present on the LHRH neurons, we evaluated the possibility that the action of cytokines could be mediated by stimulation of GABAergic neurons in the

MBH. Indeed, in the case of GMCSF, its inhibitory action on LHRH release can be partially reversed by the GABA_A receptor blocker, bicuculine, which also blocks the inhibitory action of GABA, itself, on the response of the LHRH terminals to NO. Therefore, we believe that the inhibitory action of cytokines on LHRH release is mediated by stimulation of GABA neurons (Kimura et al., 1997).

Role of NO in mating behavior

LHRH controls lordosis behavior in the female rat and is also involved in mediating male sex behavior. Studies *in vivo* have shown that NO stimulates the release of LHRH involved in inducing sex behavior. This behavior can be stimulated by third ventricular injection of NP and is blocked by inhibitors of NOS. Apparently, there are two LHRH neuronal systems: one, with axons terminating on the hypophyseal portal vessels, the other with axons terminating on neurons that mediate sex behavior (Mani et al., 1994). NO is also involved in inducing penile erection by the release of NO from NOergic neurons innervating the corpora cavernosa penis. The role of NO in sex behavior in both sexes has led us to change the name of NO to the sexual gas (McCann and Rettori, 1997).

Potential role of leptin in reproduction

The hypothesis that leptin may play an important role in reproduction stems from several findings. First, the Ob/Ob mouse, lacking the leptin gene, is infertile and has atrophic reproductive organs. Gonadotropin secretion is impaired and very sensitive to negative feedback by gonadal steroids as is the case for prepubertal animals (Swerdloff et al., 1976, 1978). It has now been shown that treatment with leptin can recover the reproductive system in the Ob/Ob mouse by leading to growth and function of the reproductive organs and fertility (Chehab et al., 1996) via secretion of gonadotropins (Barash et al., 1996).

The critical weight hypothesis of the development of puberty states that when body fat stores have reached a certain point puberty occurs (Frisch and McArthur, 1974). This hypothesis in its original form does not hold because if animals are underfed, puberty is delayed, but with access to food, rapid weight gain leads to the onset of puberty at

weights well below the critical weight under normal nutritional conditions (Ronnekleiv et al., 1978). We hypothesized that during this period of refeeding or at the time of the critical weight in the normally fed animals as the fat stores increase, there is increased release of leptin from the adipocytes into the blood stream and that this acts on the hypothalamus to stimulate the release of LHRH with resultant induction of puberty. Indeed, leptin has been found recently to induce puberty (Chehab et al., 1997).

We initiated studies on its possible effects on hypothalamic–pituitary function. We anticipated that it would also be active in adult rats and therefore studied its effect on the release of FSH and LH from hemipituitaries, and also its possible action to release LHRH from MBH explants *in vitro*. To determine if it was active *in vivo*, we used a model that we have often used to evaluate stimulatory effects of peptides on LH release; namely, the ovariectomized, estrogen-primed rat. Because our supply of leptin was limited, we began by microinjecting it into the 3V in conscious animals bearing implanted third ventricular cannulae, and also catheters in the external jugular vein extending to the right atrium, so that we could draw blood samples before and after the injection of leptin and measure the effect on plasma FSH and LH (Yu et al., 1997b).

Effect of leptin on gonadotropin release

We found that under our conditions, leptin had a bell-shaped dose-response curve to release LH from anterior pituitaries incubated *in vitro*. There was no consistent stimulation of LH release with a concentration of 10^{-5} M. Results became significant with 10^{-7} M and remained on a plateau through 10^{-11} M with reduced release at a concentration of 10^{-12} M that was no longer significant statistically. The release was not significantly less than that achieved with LHRH (4×10^{-8} M). Under these conditions, there was no additional release of LH when leptin (10^{-7} M) was incubated together with LHRH (4×10^{-8} M). In certain other experiments, there was an additive effect when leptin was incubated with LHRH; however, this effect was not uniformly seen. The results indicate that leptin was only slightly less effective to release LH, than LHRH itself (Yu et al., 1997b).

Effect of leptin on FSH release

In the incubates from these same glands, we also measured FSH release and found that it showed a similar pattern as that of LH, except that the sensitivity in terms of FSH release was much less than that for LH. The minimal effective dose for FSH was 10^{-9} M, whereas it was 10^{-11} M for LH. The responses were roughly of the same magnitude at the effective concentrations as obtained with LH and the responses were clearly equivalent to those observed with 4×10^{-9} M LHRH. Combination of LHRH with a concentration of leptin which was just below significance gave a clear additive effect (Yu et al., 1997b). The action of leptin to stimulate both LH and FSH release was mediated by the long form of the leptin receptor that is located on the cell surface of the gonadotropes. The mechanism of action is the same as that of FSHRH and LHRH, the only difference is that the action is mediated by leptin receptors that increase intracellular free Ca^{2+} activating nNOS that generates NO that activates GC followed by PKG leading to extrusion of FSH and LH secretory granules from the gonadotropes (Fig. 2).

Effect of leptin on LHRH release

There was no significant effect of leptin in a concentration range of 10^{-6} – 10^{-12} M on LHRH release during the first 30 min of incubation; however, during the second 30 min, the highest concentration produced a borderline significant decrease in LHRH release with 10^{-6} M, followed by a tendency to increase with lower concentrations and a significant ($P < 0.01$), plateaued increase with the lowest concentrations tested 10^{-10} and 10^{-12} M (57). Both the FSH and LH-releasing actions of leptin were blocked by NMMA indicating that NO mediates its action (Yu et al., 1997b).

The effect of intraventricularly injected leptin on plasma gonadotropin concentrations in ovariectomized, estrogen-primed rats

The injection of the diluent for leptin into the 3V (Krebs-Ringer Bicarbonate, 5 μ l) had no effect on pulsatile FSH or LH release, but the injection of leptin (10 μ g) uniformly produced an increase in

plasma LH with a variable time-lag ranging from 10 to 50 minutes, so that the maximal increase in LH from the starting value was highly significant ($P < 0.01$) and constituted a mean increase of 60% above the initial concentration. In contrast, leptin inhibited FSH release on comparison with the results with the diluent, but the effect was delayed and occurred mostly in the second hour. Therefore, at this dose of estrogen (10 μ g estradiol benzoates, 72 h before experiments), leptin stimulates the release of LHRH and inhibits the release of FSHRH (Walczewska et al., 1999).

Mechanism of action of leptin on the hypothalamic pituitary axis

We have shown that leptin exerts its action at both hypothalamic and pituitary level by activating NOS since its effect to release LHRH, FSH and LH in vitro (Yu et al., 1997b) is blocked by NMMA. Leptin, in essence, is a cytokine secreted by the adipocytes. It, like the cytokines, seems to reach the brain via a transport mechanism mediated by the Ob/Ob_a receptors (Cioffi et al., 1996) in the choroid plexus (Schwartz et al., 1996). These receptors have an extensive extracellular domain, but a greatly truncated intracellular domain (Cioffi et al., 1996) and mediate transport of the cytokine by a saturable mechanism (Banks et al., 1996). Following uptake into the cerebrospinal fluid (CSF) through the choroid plexus, leptin is carried by the flow of CSF to the 3V, where it either diffuses into the hypothalamus through the ependymal layer lining the ventricle or combines with Ob/Ob_a (Cioffi et al., 1996) receptors on terminals of responsive neurons that extend to the ventricular wall.

The Ob/Ob_b receptor has a large intracellular domain that presumably mediates the action of the protein (Schwartz et al., 1996). These receptors are wide-spread throughout the brain (Schwartz et al., 1996), but particularly localized in the region of the paraventricular (PVN) and arcuate nuclei (AN). Leptin activates stat 3 within 30 min after its intraventricular injection (Vaisse et al., 1996). Stat 3 is a protein that is important in conveying information to the nucleus to initiate DNA-directed messenger ribonucleic acid (mRNA) synthesis. Following injection of bacterial lipopolysaccharide (LPS), it is

also activated, but in this case, the time delay is 90 min presumably because LPS has been shown to induce IL-1 beta (β) mRNA in the same areas — namely, the PVN and AN (Schwartz et al., 1996), IL-1 β mRNA would then cause production of IL-1 β that would activate stat 3. On entrance into the nucleus, stat 3 would activate or inhibit DNA-directed mRNA synthesis. In the case of leptin, it activates corticotropin-releasing hormone (CRH) mRNA in the PVN, whereas in the AN, it inhibits neuropeptide Y (NPY) mRNA resulting in increased CRH synthesis and presumably release in the PVN and decreased NPY synthesis and release in the AN (Schwartz et al., 1996). Presumably, the combination of leptin with these transducing receptors also either increases or decreases the firing rate of that particular neuron. In the case of the AN-median eminence area, leptin may enter the median eminence by diffusion between the tanycytes or alternatively by combining with its receptors on terminals of neurons projecting to the tanycytes. Activation of these neurons would induce LHRH release.

The complete pathway of leptin action in the MBH to stimulate LHRH release is not yet elucidated. Arcuate neurons bearing Ob/Ob receptors may project to the median eminence to the tanycyte/portal capillary junction. Leptin would either combine with its receptors on the terminals that transmit information to the cell bodies in the AN or diffuse to the AN to combine with its receptors on the perikarya of AN neurons. Because leptin decreases NPY mRNA and presumably NPY biosynthesis in NPY neurons in the AN (Chehab et al., 1997), we postulate that leptin causes a decrease in NPY release. Because NPY inhibited LH release in intact and castrated male rats (Reznikov and McCann, 1993), we hypothesize that NPY decreases the release of LHRH by inhibiting the noradrenergic neurons which mediate pulsatile release of LHRH. Therefore, when the release of NPY is inhibited by leptin, noradrenergic impulses are generated, that act on α_1 receptor on the NOergic neurons causing the release of NO which diffuses to the LHRH terminals and activates LHRH release by activating guanylate cyclase and cyclooxygenase₁ as shown in our prior experiments reviewed above. Leptin acts to activate NOS as indicated because its release of LHRH is blocked by inhibition of NOS (Yu et al., 1997b).

The LHRH enters the portal vessels and is carried to the anterior pituitary gland where it acts to stimulate FSH and particularly LH release by combining with its receptors on the gonadotropes. The release of LH and to a lesser extent FSH is further increased by the direct action of leptin on its receptors in the pituitary gland (Cioffi et al., 1996; Naivar et al., 1996; Yu et al., 1997b).

We hypothesize that leptin may be a critical factor in induction of puberty as the animal nears the so-called critical weight. Either metabolic signals reaching the adipocytes, or signals related to their content of fat cause the release of leptin, which increases LHRH and gonadotropin release, thereby initiating puberty and finally ovulation and onset of menstrual cycles. In the male, the system would work similarly; however, there is no preovulatory LH surge brought about by the positive feedback of estradiol. Sensitivity to leptin is undoubtedly under steroid control and we are actively working to elucidate this problem.

During fasting, the leptin signal is removed and LH pulsatility and reproductive function decline quite rapidly. In women with anorexia nervosa, this causes a reversion to the prepubertal state, which can be reversed by feeding. Thus, leptin would have a powerful influence on reproduction throughout the reproductive lifespan of the individual. The consequences to gonadotropin secretion of overproduction of leptin, as has already been demonstrated in human obesity, are not clear. There are often reproductive abnormalities in this circumstance and whether they are due to excess leptin production or other factors, remains to be determined. In conclusion, it is now clear that leptin plays an important role in control of reproduction by actions on the hypothalamus and pituitary.

Abbreviations

3V	third ventricle
AN	arcuate nucleus
c	chicken
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
CRH	corticotropin-releasing hormone
CSF	cerebrospinal fluid
FSH	follicle-stimulating hormone
GA	lutamic acid

GABA	gamma amino butyric acid
GMCSF	granulocyte macrophage colony-stimulating factor
GnRH	gonadotropin-releasing hormone
GnRHR	GnRH receptor
GTP	guanosine triphosphate
i	inducible
IL-1 α	interleukin-1 alpha
l	lamprey
LPS	lipopolysaccharide
m	mammalian
MBH	medial basal hypothalami
mLHRH	Mammalian luteinizing hormone-releasing hormone
mRNA	messenger ribonucleic acid
NE	norepinephrine
NMMA	N ^G -monomethyl-L-arginine
nNOS	neural nitric oxide synthase
NP	nitroprusside
NPY	neuropeptide Y
OVL	organum vasculosum lamina terminalis
PGE ₂	prostaglandin E ₂
PLA ₂	phospholipase A ₂
PVN	paraventricular nucleus

Acknowledgements

This work was supported by NIH Grant MH51853. We would like to thank Judy Scott and Natasha Hunter for their excellent secretarial support.

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Gonadotropin-releasing hormone (GnRH) surge generator in female rats

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Introduction

In rats, gonadotropin-releasing hormone (GnRH) expressing neurons, which control luteinizing hormone (LH) and follicle stimulating hormone secretions from the anterior pituitary, are mainly located in the preoptic area (POA) (for review see Silverman et al., 1994), but some GnRH neurons are also found in the mediobasal hypothalamus (MBH) in rats (Kawano and Daikoku, 1981; King et al., 1982; Shivers et al., 1983; Merchenthaler et al., 1984). However, the GnRH neurons found in the MBH are not located in the arcuate nucleus but in the lateral hypothalamic area, close to the dorso-medial surface of the optic tract and the base of the hypothalamus (Funabashi et al., 1997a,b). These GnRH neurons in the MBH are involved in the GnRH pulse generator, which is not described in detail in this chapter. At any rate, this wide and scattered distribution of GnRH neurons in the brain makes study of the physiology of GnRH neurons difficult.

One of most remarkable characteristic of GnRH neurons is that they do not originate from the hy-

pothalamus but from the olfactory placode and migrate toward the hypothalamus during developmental stages (Schwanzel-Fukuda et al., 1992). Another interesting characteristic is their secretion patterns. Secretion of LH, which is assumed to reflect GnRH secretion from the hypothalamus into the portal vein, shows two remarkably different patterns. One is pulsatile LH secretion, and another is pre-ovulatory LH surge secretion. The fact that these secretory patterns reflect those of GnRH has now been determined; the pulsatile LH secretion is followed by pulsatile GnRH secretion in rats (Levine and Ramirez, 1982), sheep (Clarke and Cummins, 1982) and monkeys (Woller et al., 1992), and the surge of LH secretion is followed by a surge of GnRH secretion in rats (Sarkar et al., 1976), sheep (Caraty et al., 1989; Moenter et al., 1992), and monkeys (Levine et al., 1985; Pau et al., 1993).

We have hypothesized that these two patterns of GnRH secretion are controlled by different neural mechanisms (for review see Kimura and Funabashi, 1998): the GnRH pulse generator, previously known as the tonic center, and the GnRH surge generator, previously known as the phasic or the cyclic center (Gorski, 1971). Classical neuroendocrinological studies indicate that in female rats, the neuronal component responsible for inducing the LH surge is located in the POA (Everett, 1965; Halász and Gorski, 1967; Gorski, 1971; Kawakami et al., 1971). According to our hypothesis, which includes this tra-

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ditional concept, the GnRH pulse generator located in the MBH makes intimate contact with opioid neurons that mediate the negative feedback effects of estrogen and the GnRH surge generator located in the POA involves GABA neurons and arginine-vasopressin (AVP) neurons in addition to GnRH neurons. In this chapter, we focus on the putative GnRH surge generator of female rats (Funabashi et al., 2002).

Ultra-short feedback of GnRH

It has now been established, by checking GnRH secretion, that the surge of LH secretion is produced not only by increasing the responsiveness of the anterior pituitary to GnRH but also by increasing GnRH secreted into the portal blood as the result of a positive feedback action of estrogen, which is administered at a certain concentration and duration to the hypothalamus and the pituitary gland (Kalra, 1993; Freeman, 1994). We were first interested in how this pattern of GnRH secretion occurs. Since the bulk of GnRH secretion, the GnRH surge, occurs during a certain period and for a certain duration (Sarkar et al., 1976; Levine et al., 1985), many GnRH neurons need to be excited at the same time. Several lines of morphological evidence indicate that GnRH neurons in the POA make synaptic contacts with each other (Merchenthaler et al., 1984; Leranthe et al., 1985; Pelletier, 1987), suggesting that GnRH affects GnRH neurons in the POA. In support of this, electrophysiological studies revealed both excitatory and inhibitory effects of GnRH on neurons in the POA (Moss, 1979). Hence, we thought that an ultra-short feedback mechanism (Hyypä et al., 1971) is involved in the bulk of GnRH secretion, resulting in a surge of LH secretion. To this end, we injected GnRH into the POA in estrogen-primed ovariectomized (OVX) or proestrous rats. We found that the injection potentiated and advanced the timing of the LH surge (Hiruma et al., 1989), while the injection of GnRH antagonist delayed the LH surge (Funabashi et al., 1994). In accord with these results, it was also reported that the injection of GnRH antagonist into the POA disrupted the estrous cyclicity in rats (Weesner and Pfaff, 1994). However, injection of neither GnRH nor GnRH antagonist into the POA affected the pulsatile LH secretion in OVX rats, sug-

gesting that ultra-short feedback mechanism in the POA is only acting for the GnRH surge generator but not for the GnRH pulse generator (Funabashi et al., 1994). It was recently reported that GnRH receptor mRNA was expressed in the POA (Han et al., 1999). Thus, a stimulatory ultra-short feedback of GnRH in the POA is involved in inducing the GnRH surge.

GABA and LH surge

To understand the mechanism underlying the generation of the GnRH surge, it is necessary to determine the neuronal components of the GnRH surge generator. There is increasing evidence indicating that GABAergic inputs are profoundly involved in regulating the surge of LH secretion (Demling et al., 1985; Adler and Crowley, 1986; Donoso and Banzan, 1986; Flügge et al., 1986; Herbison and Dyer, 1991; Jarry et al., 1995; Mitsushima et al., 1997). Further, it has been shown that GnRH neurons in the POA express subunits of GABA_A receptor (Petersen et al., 1993a; Jung et al., 1998; Sim et al., 2000) and respond to bicuculline, a GABA_A receptor antagonist (Sim et al., 2000). This means that a decrease in inhibitory GABA tone on GnRH neurons results in an activation of GnRH neurons. Consistent with this, we have shown that the intravenous infusion of bicuculline, a GABA_A receptor antagonist, on the morning of proestrus induces a premature surge-like secretion of LH (Kimura and Jinnai, 1994). We then wondered what similarities there were between the spontaneous LH surge and the bicuculline-induced surge-like LH secretion.

Since it was reported that GnRH neurons within the POA express c-Fos protein in association with the LH surge on the day of proestrus (Lee et al., 1990a) and in ovariectomized estrogen- and progesterone-primed female rats (Lee et al., 1990b), we examined whether the infusion of bicuculline on the day of proestrus advances the timing of c-Fos expression in GnRH neurons as well as the surge of LH secretion. We thought that if the premature surge-like LH secretion was due to the advancement of activation of GnRH neurons in the surge generator, GnRH neurons would express c-Fos at an early time when c-Fos expression is usually not observed in proestrous rats. We thus used double immunocyto-

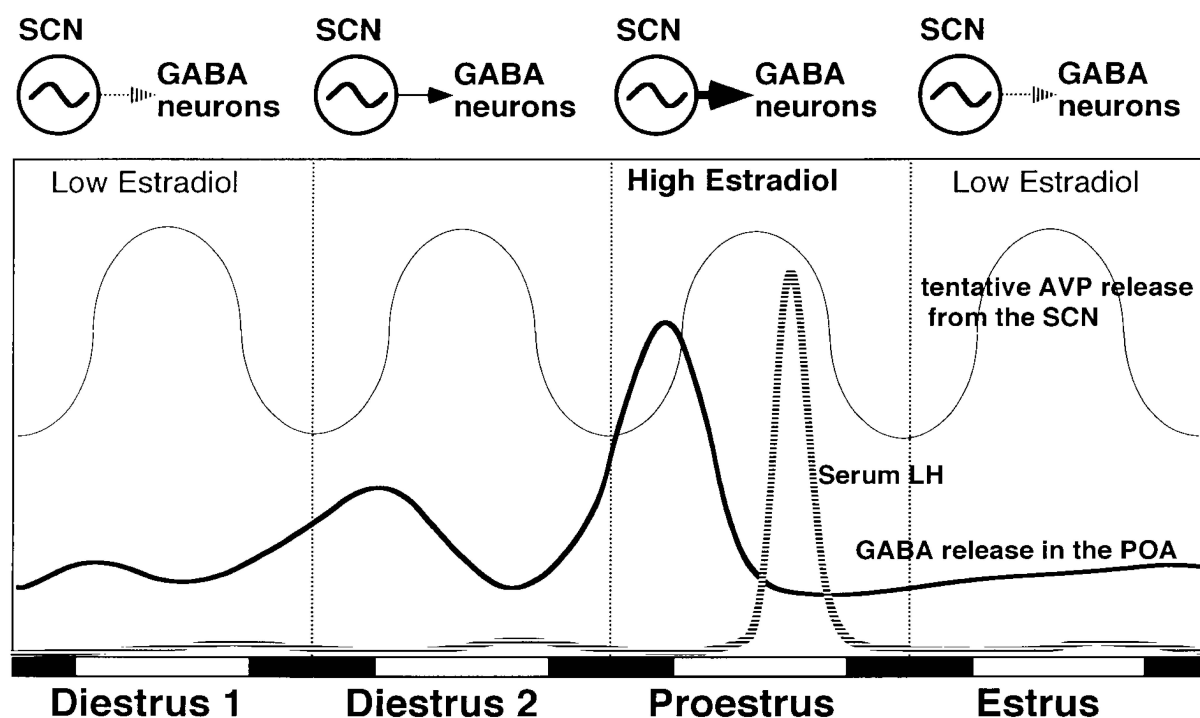


Fig. 1. Schematic illustration showing the relationship among GnRH, GABA and AVP secretions during the estrous cycle in rats. The activity of AVP neurons in the SCN is constantly exhibiting a circadian during the estrous cycle, based on changes in AVP release (Shinohara et al., 1994; Kalsbeek et al., 1995) and its mRNA in the SCN (Krajnak et al., 1998a,b). Since serum estrogen levels are low in diestrus 1 and 2, and thus levels of AVP 1a receptors are low in the POA, circadian signals are not transmitted to GnRH neurons or GABA neurons. Inhibition of GABA neurons on GnRH neurons develops as a function of levels of serum estrogen. An increase in serum estrogen also elicits the expression of AVP 1a receptor in the POA. This makes it possible for GnRH neurons or GABA neurons to communicate with AVP neurons coming from the SCN. Disinhibition of GABA neurons on GnRH neurons that occurs during the critical period is probably permissive for activation of GnRH neurons. Clock information conveyed through AVP neurons may inhibit GABA activity or activate GnRH neurons. Hence, the LH surge is induced as the result of positive feedback effects of estrogen.

chemistry for c-Fos and GnRH (Funabashi et al., 1997a). As expected, c-Fos was expressed in approximately 50% of GnRH neurons in saline-infused rats killed in the late afternoon but not in the saline-infused rats killed in the early afternoon. Similarly, approximately 50% of GnRH neurons in bicuculline-infused rats killed in the early afternoon were found to express c-Fos. The distribution and proportion of GnRH neurons expressing c-Fos in response to bicuculline were identical to those during the spontaneous LH surge as observed in saline-injected rats killed in the late afternoon. These results indicate that bicuculline infusions activate GnRH neurons. This activation of GnRH neurons would account for the observed increase in serum LH, because the intensity of c-Fos expression in GnRH neurons is

closely associated with the amount of LH secreted in proestrous rats (Lee et al., 1992). Therefore, we conclude that bicuculline advances the timing of the activation of GnRH neurons. This provides additional evidence that GABAergic neurons are involved in the GnRH surge generator and subsequently the LH surge. It was reported that GABA release in the POA seemed to decline prior to the onset of a surge-like secretion of LH in Ovariectomized estrogen-primed rats (Jarry et al., 1992, 1995). Quite recently, we determined the GABA release in the POA during the estrous cycle by microdialysis (Fig. 1). We found that the GABA release markedly increased from late in the night of diestrus 2 through the morning of proestrus, when it attained its peak, and thereafter it declined sharply until the critical period of proestrus.

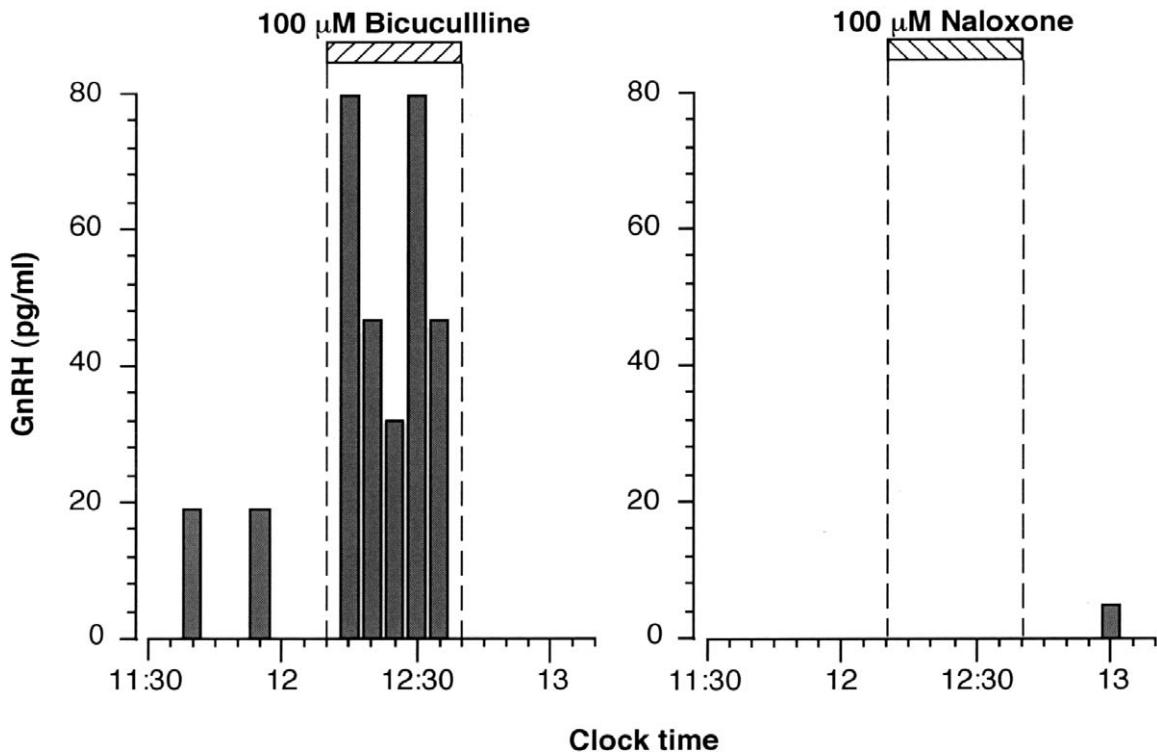


Fig. 2. Effects of bicuculline and naloxone on the GnRH secretion in POA slices derived from proestrous rats. On the morning of proestrus, rats were killed and 3 200- μ m-thick POA slices were cut with a brain slicer. After 1 h of incubation in a standard artificial cerebrospinal fluid (aCSF) with 10 mM glucose at a flow rate of 500 μ l/min, 50 μ l of aCSF were collected at 5-min intervals. Concentrations of GnRH were determined by EIA. As shown here, 100 μ M bicuculline infusion markedly increased the GnRH secretion but 100 μ M naloxone was without effect. This preliminary result suggests that a decrease in GABA tone in the POA elicits GnRH secretion but that a decrease in opioid tone does not.

This suggests that the preovulatory elevation of the GABA release from the night through to the morning of proestrus, followed by a sharp decline, is closely associated with the onset of the preovulatory LH surge in cyclic female rats (Mitsushima et al., 2002). This also provides strong evidence that a decrease in GABA tone in the POA triggers the activation of GnRH neurons. We further confirmed this concept by checking changes in GnRH release from POA slices. When 100 μ M bicuculline was applied for 30 min, GnRH release was markedly increased in 200 μ m thick slices obtained from a proestrous rat, but naloxone had no significant effect on GnRH release (Fig. 2). Although this is a preliminary result, it shows that disinhibition of GABA, but not of opioid, on GnRH neurons elicits a surge-like GnRH secretion from the POA, as we have suggested that opioid neurons are not involved in the LH surge (Kimura

and Jinnai, 1994; Funabashi et al., 1997b; Kimura and Funabashi, 1998).

The suprachiasmatic nucleus (SCN), POA, and the GnRH surge generator

A circadian clock located in the SCN (Meijer and Rietveld, 1989) is involved in the timing of the LH surge in rats (Wiegand et al., 1980; van der Beek, 1996). This is demonstrated clearly by the fact that a daily afternoon rise of LH secretion occurs if ovariectomized rats are treated with a certain amount of estrogen (Everett and Sawyer, 1950; Legan and Karsch, 1975), indicating that the neuronal component responsible for the GnRH surge in the POA develops receptiveness to clock information from the SCN (Wiegand et al., 1978) along with the action of estrogen (Petersen et al., 1993b). It is therefore nec-

essary to know which neurons in the SCN send the circadian clock information to the GnRH surge generator in the POA. Since arginine-vasopressin (AVP) and vasoactive intestinal polypeptide (VIP) neurons provide the major output from the SCN (Watts and Swanson, 1987), these two peptides are the most likely candidates (Harney et al., 1996; Krajnak et al., 1998a,b).

To determine whether the SCN can drive the circadian secretion of GnRH from neurons in the POA and which neurons mediate the clock information to the GnRH surge generator in the POA, we measured the secretion of GnRH, AVP and VIP in co-cultures of the POA and the SCN at 2-h intervals over a period of 120 hours (Funabashi et al., 2000a). Co-cultures were treated with antimitotics, because such treatment alters the phase relationship between AVP and VIP rhythms, causing the periods of the two peptide rhythms to diverge from each other (Shinohara et al., 1995).

We found that the secretion of GnRH in co-cultures exhibited a significant circadian rhythm in the presence of estrogen but not in the absence of estrogen. In each co-culture, the period of the GnRH circadian rhythm was the same as that of the AVP circadian rhythm, but different from the VIP circadian rhythm. As mentioned above, the circadian rhythm of the AVP release differed from that of VIP release because of the antimitotic treatment. Furthermore, the peak phase of the GnRH rhythm occurred at the time same as that of the AVP rhythm. However, the peak phase of the GnRH rhythm was not always the same as that of the VIP rhythm. The administration of AVP significantly increased GnRH secretion in individual POA cultures in the presence of estrogen, but the administration of VIP had no effect. These results suggest that, in co-cultures of the SCN and the POA, AVP neurons drive the circadian secretion of GnRH in the presence of estrogen. We therefore conclude that AVP neurons in the SCN mediate circadian clock information to the GnRH surge generator in the POA. In support of this conclusion, the injection of AVP receptor antagonist at 1300 h in proestrous rats resulted in significant decreases in both LH and prolactin secretion (Funabashi et al., 1999). Furthermore, Palm et al. reported that when AVP was injected into the POA, AVP stimulated the LH surge (Palm et al., 1999, 2001). These results

provide further evidence supporting our hypothesis that endogenous AVP plays a stimulatory role in the induction of the surge of LH.

On the basis of these findings, we searched for the site of estrogen action in association with the LH surge. We thought that estrogen may target neurons expressing AVP receptor in the POA. We therefore tried to determine whether estrogen affected the expression of AVP receptor mRNA in the POA of female rats (Funabashi et al., 2000b). By reverse transcription-PCR, we found that all three types of the AVP receptor mRNA, V1a, V1b, and V2, were expressed in the POA, though the amount of PCR products was apparently different among them. Using *in situ* hybridization histochemistry, we found that AVP V1a receptor mRNA was expressed in the POA, especially in the anteroventral periventricular nucleus of the POA. This was in good accord with a previous report (Ostrowski et al., 1994). In contrast, AVP V1b and V2 receptor mRNAs were not abundant in this area. Northern blot analysis revealed that estrogen significantly increased the expression of AVP V1a receptor mRNA in the POA of young ovariectomized female rats.

Can POA time the LH surge without the SCN?

As described above, the SCN is important for timing the LH surge at least in rats. However, we cannot generalize this concept for rats to other species. For example, the surge of LH secretion occurs as a function of time after estrogen priming in sheep (Goodman et al., 1981). But how does the sheep brain count the time after estrogen treatment without a clock? Is the SCN essential for inducing the LH surge in rats? To answer this question, we examined whether GnRH neurons by themselves can release GnRH as a function of time after estrogen treatment. To this end, we measured GnRH release in cultures of GT1-7 cells after applying serum shock, which has been shown to induce a circadian rhythm in another cell lines (Balsalobre et al., 1998; Akashi and Nishida, 2000). As a result, a bulk release of GnRH was observed 12 h after the serum shock (Fig. 3). This preliminary result suggests that an increase in GnRH secretion can be induced by a certain stimulation without any other synaptic input neurons. It needs to be determined, however, whether

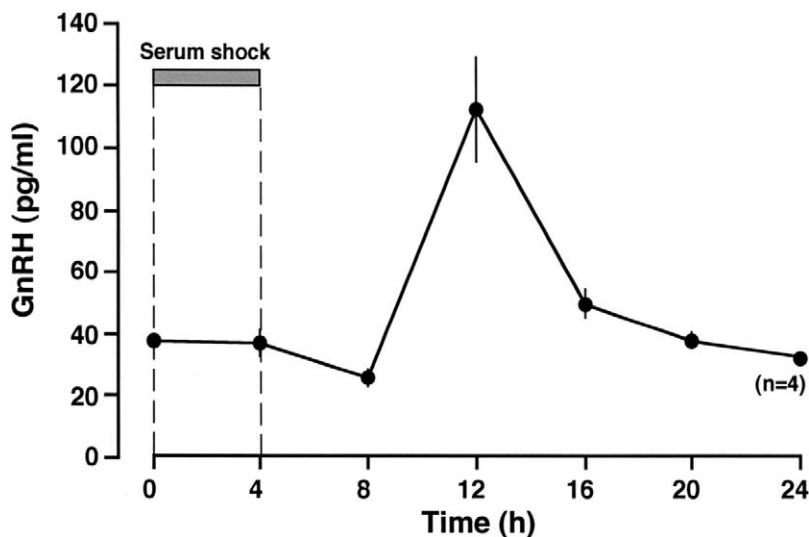


Fig. 3. GT1-7 cells were cultured and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) in an atmosphere containing 5% CO₂ at 37°C. When they reached 60–80% confluency, GT1-7 cells were dissociated and the cell suspension (5×10^4) was plated in 60 mm dishes. These cells were maintained in the D-MEM/F12 medium supplemented with 10% FBS. Then the medium was replaced with one containing 50% FBS (serum shock). Thereafter, all medium was removed and replaced with fresh medium containing 2% FBS at 4-h intervals. Concentration of GnRH was measured in 50 μ l of medium. Data are shown as the mean \pm SEM ($n = 4$). As shown here, a bolus of GnRH was induced by serum shock.

such increase in GnRH secretion resembles the surge of GnRH secretion, or, most importantly, whether estrogen can produce the same effect as the serum shock.

Acknowledgements

We are grateful to Dr. K. Wakabayashi and the NIDDK for providing radioimmunoassay materials. We also thank Dr. T. Nett for antibody to GnRH (CR-R11B71). GT1-7 cells were generously donated by Drs R.I. Weiner and P.L. Mellon. Part of the present study was supported by a Grant-in-Aid for Scientific Research (C:11670075 to FK and C:11680790 to TF) and for Exploratory Research (13877011 to FK) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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Mechanisms of inhibition of LHRH release by alcohol and cannabinoids

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Introduction

This paper will review our research and that of our associates on the effects of alcohol and cannabinoids on reproduction. Today, alcohol is classified as a psychotropic drug, like delta-9 tetrahydrocannabinol (THC), the active ingredient of marihuana. A large number of neurotransmitters not only the classical ones, but also a host of neuropeptides that can act as neurotransmitters or neuromodulators, exist in the central nervous system (CNS) and there is abundant evidence that alcohol and cannabinoids can affect a number of them. Therefore, mechanisms of action of these drugs in the CNS are very complex.

Addiction to alcohol or marihuana produces numerous deleterious effects in the organism. Among these alterations is the suppression of reproduction in humans, monkeys and small rodents by inhibition of the release of luteinizing hormone (LH). This inhibition of LH secretion is caused mainly by hypothalamic action to inhibit the release of luteinizing hormone-releasing hormone (LHRH), in vivo and in vitro. In conscious, ovariectomized rats, intragastric administration of alcohol (3 g/kg), a dose that

causes mild intoxication, has been shown to produce a marked decrease in plasma LH concentrations on comparison with the unaffected LH values of diluent administered animals (Dees et al., 1985). There was a highly significant decrease in the area under the secretion curve of LH, with a reduction of LH pulses. On the other hand, following a challenge with exogenous LHRH, the response of LH was the same as in controls, indicating that pituitary responsiveness was the same for alcohol and saline groups. In contrast to LH, alcohol did not significantly alter pulsatile FSH secretion, indicating that alcohol selectively inhibited pulsatile release of LHRH but not the putative FSHRF. Furthermore, the secretion of LH by pituitaries incubated in vitro in the presence of different concentrations of alcohol (50–100 mM) was the same as without alcohol.

Similar results were obtained when THC was injected into the third cerebral ventricle. A single dose of THC (2 μ l of 10^{-6} M) significantly decreased serum LH levels as compared to values in vehicle injected rats (Wenger et al., 1987). Also, there was no effect on plasma FSH levels as seen with alcohol. Furthermore, the response to a challenge dose of LHRH on LH secretion by cultured dispersed pituitary cells was the same in the presence of THC or vehicle. Therefore, we investigated the effect of both drugs on LHRH release from medial basal hypothalamus (MBH) incubated in an in vitro system.

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Effect of alcohol on LHRH release

To understand the inhibitory pathways involved in the inhibition of LHRH release by alcohol, it is necessary to put them into the context of our previous work on the NOergic control of LHRH release. Our previous work indicated that the release of LHRH is controlled by nitric oxide (NO) (Rettori et al., 1993; Canteros et al., 1996). NO is formed by conversion of arginine to citrulline and NO in equimolar concentrations by the action of nitric oxide synthase (NOS). There are three isoforms of NOS, neuronal NOS (nNOS) and endothelial NOS (eNOS) are constitutive, and need the presence of Ca^{2+} to form NO, the inducible NOS (iNOS) is Ca^{2+} independent and is induced mostly by endotoxins as well by cytokines (Moncada et al., 1991). In the present work we will be referring to constitutive NOS, mainly to nNOS. nNOS has been demonstrated by immunocytochemical methods in neurons in some areas of the CNS including some regions of hypothalamus, as the median eminence-arcuate region. Previous research has indicated that NO stimulates the release of LHRH both in vivo and in vitro. On the basis of in vitro experiments using incubations of MBH in a static incubation system, it has been determined that norepinephrine (NE) activates constitutive NOS in this region. The NO released from these neurons diffuses to LHRH terminals, where it induces the release of LHRH. It has been shown that NO not only activates guanylate cyclase followed by increased cyclic guanosine monophosphate (cGMP) release but also activates cyclooxygenase (COX) that increases release of eicosanoids (Rettori et al., 1992). Prostaglandin E₂ (PGE₂) by activating adenylate cyclase (Ojeda et al., 1979) with consequent increase in cyclic adenosine monophosphate (cAMP) evokes exocytosis of LHRH granules by activation of protein kinase A. The LHRH released diffuses into the hypophyseal portal vessels that deliver it to the anterior pituitary gland where it acts on gonadotropes to release LH. Support for this theoretical pathway stems from the ability of inhibitors of NOS, such as NG-monomethyl-L-arginine, to inhibit LHRH release, whereas releasers of NO, such as sodium nitroprusside (NP), induce LHRH release as well as that of PGE₂ from MBH (Rettori et al., 1992).

The release of LHRH is not only under the control of stimulatory neurotransmitters such as NE (Rettori

et al., 1993) and glutamic acid (Rettori et al., 1994) via NO, but is also under the control of inhibitory neurotransmitters such as gamma-amino butyric acid (GABA) (Masotto et al., 1989) and beta-endorphin (Lomniczi et al., 2000) both of which inhibit LHRH release. The inhibitory action of GABA on LHRH release could be prevented by hemoglobin (a scavenger of NO), indicating that NO has a stimulatory action on GABA release. It is possible that the increase in GABA release during LHRH secretion induced by NO could be a mechanism to terminate the pulses of LHRH (Seilicovich et al., 1995).

Beta-endorphin also can inhibit LHRH release, probably by stimulating μ -opiate receptors on NO-ergic neurons because we have shown that beta-endorphin inhibits the activity of NOS in MBH, whereas naltrexone, a μ -opiate receptor antagonist, increased the activity of NOS in this tissue. Beta-endorphin also blocked the action of sodium nitroprussiate (NP) (a NO donor) on PGE₂ release and consequently LHRH secretion (Faletti et al., 1999).

Since alcohol has been shown to increase the release of GABA and of beta-endorphin we studied the interactions between GABA, beta-endorphin and alcohol. We confirmed that alcohol increases the release of GABA and beta-endorphin. Furthermore, beta-endorphin also stimulated GABA release, but GABA had no stimulatory action on beta-endorphin release and NP significantly inhibited the release of beta-endorphin from MBH in our in vitro experimental model. Furthermore, alcohol diminished significantly the N-methyl-D-aspartate (NMDA) stimulated NOS and this inhibition could be reversed by addition of naltrexone and bicuculline (a GABA-A receptor antagonist). However, bicuculline (10^{-4} M) could reverse the alcohol induced block of NMDA-stimulated LHRH release only when the concentration of alcohol used was 50 mM. At a higher concentration of alcohol such as 100 mM, the inhibitory action of alcohol could be reversed by naltrexone (10^{-6} M) and not by bicuculline. These results suggest that the primary effect of alcohol is to stimulate beta-endorphin release which in turn stimulates GABA release. Both of these inhibitory neurotransmitters then act together to suppress LHRH release. Since beta-endorphin decreases NOS activity while GABA and alcohol are without effect on NOS activity, they are acting down stream from NOS that is,

they block the NOergic activation of cyclooxygenase (COX), that is necessary for LHRH release. This conclusion was confirmed, since the addition of an effective concentration of 8-bromo cGMP (a stable analogue of cGMP) to correct for a possible blockade of guanylate cyclase did not reverse the action of GABA or alcohol (Lomniczi et al., 2000). Addition of arachidonate to provide more substrate for COX also failed to reverse this inhibition, suggesting that the primary action of both, GABA and alcohol was by inhibition of COX. This conclusion is supported also by the fact that addition of PGE₂ reversed the alcohol block on LHRH release. Recent preliminary results show that alcohol not only inhibits the activity of COX as measured by radio-conversion assay of ¹⁴C-arachidonic acid to eicosanoids, such as PGE₂ (Canteros et al., 1995) but also decreases COX content in MBH (unpublished observation from a collaborative work with V. Svivaslava and W.L. Dees). In Fig. 1 we present the diagrammatic representation of the postulated mechanism of action of alcohol to suppress NMDA-stimulated LHRH release that we described above.

Effect of the active cannabinoid, delta-9-tetrahydrocannabinol (THC) on LHRH release

All previous studies including ours in several species (Ayalon et al., 1977; Almirea et al., 1983; Wenger et al., 1987) indicate that the inhibitory effect of THC on the reproductive axis is exerted mainly at the hypothalamic level with the inhibition mainly of LH secretion by the pituitary and consequent alteration of reproductive function. Studies performed by our group (Rettori et al., 1990) using a static incubation system to incubate medial basal hypothalamic (MBH) explants in the presence of different concentrations of THC (10^{-11} to 10^{-8} M) showed that THC was without effect on basal release of LHRH. Since it was reported previously (Negro-Vilar et al., 1979), that catecholamines stimulate LHRH release, we used this approach to study the effect of THC on stimulated LHRH release and found as expected, that norepinephrine (NE) (5×10^{-5}) as well as dopamine (5×10^{-5} M) stimulated significantly the release of LHRH and that it was inhibited by the addition of THC (10^{-8} M). Since it is also established that PGE₂ is stimulated by NE and is also part of the

secretory pathway of LHRH release, we measured the release of PGE₂ from MBH in the presence of THC and found that THC (10^{-7} M) lowered significantly the release of PGE₂ from MBH as measured by RIA. The inhibition of PGE₂ release could also be due to an inhibition of COX activity. Therefore, we performed radio-conversion studies using ¹⁴C-arachidonic acid and measured the eicosanoids that are formed by the action of COX and found that addition of THC to MBH incubated with ¹⁴C-arachidonic acid had a dramatic inhibitory effect on COX activity, since all the eicosanoids measured such as 6-keto F_{1 α} , PGF_{2 α} , PGE₂ and TxB₂ were highly significantly inhibited by THC (10^{-8} and 10^{-7} M) as compared to values in controls (Rettori et al., 1990).

Our findings are in agreement with the known fact that THC and other cannabinoids inhibit adenylyl cyclase in a reversible, dose-dependent and stereoselective manner (Bidau-Russell et al., 1990). These facts taken together (inhibition of PGE₂ and PGE₂-stimulated cAMP release) explain the inhibition of LHRH release with the consequent lowering of plasma LH levels.

The effects of THC as well as of other cannabinoids were believed to be due to a non-specific interaction with the membrane lipids, since cannabinoids are highly lipophilic molecules. But since the discovery of cannabinoid receptor CB1 in the brain (Devane et al., 1988), its abundance and anatomical localization, together with the behavioral effects of THC provide the molecular basis for the action of cannabinoids. Till now, two cannabinoid receptors: CB1 localized mostly in the brain (Herkenham et al., 1990) and CB2 receptor localized mostly in peripheral tissues and immune cells (Galiègue et al., 1995) have been described. Since then, complementary DNAs have been cloned, the expression of their genes, and their functional domains have been described. The structure of the CB1 receptor exhibits the basic structure of a G-protein-coupled receptor with a molecular weight of 64 kDa (Ameri, 1999). The distribution of CB1 has been well described in rat brain by Herkenham et al. (1991) who found that these binding sites are not homogeneously distributed. Although they present high density binding by radioautographic method in some areas such as hippocampus, the hypothalamus present sparse binding that is slightly elevated in the ventromedial nu-

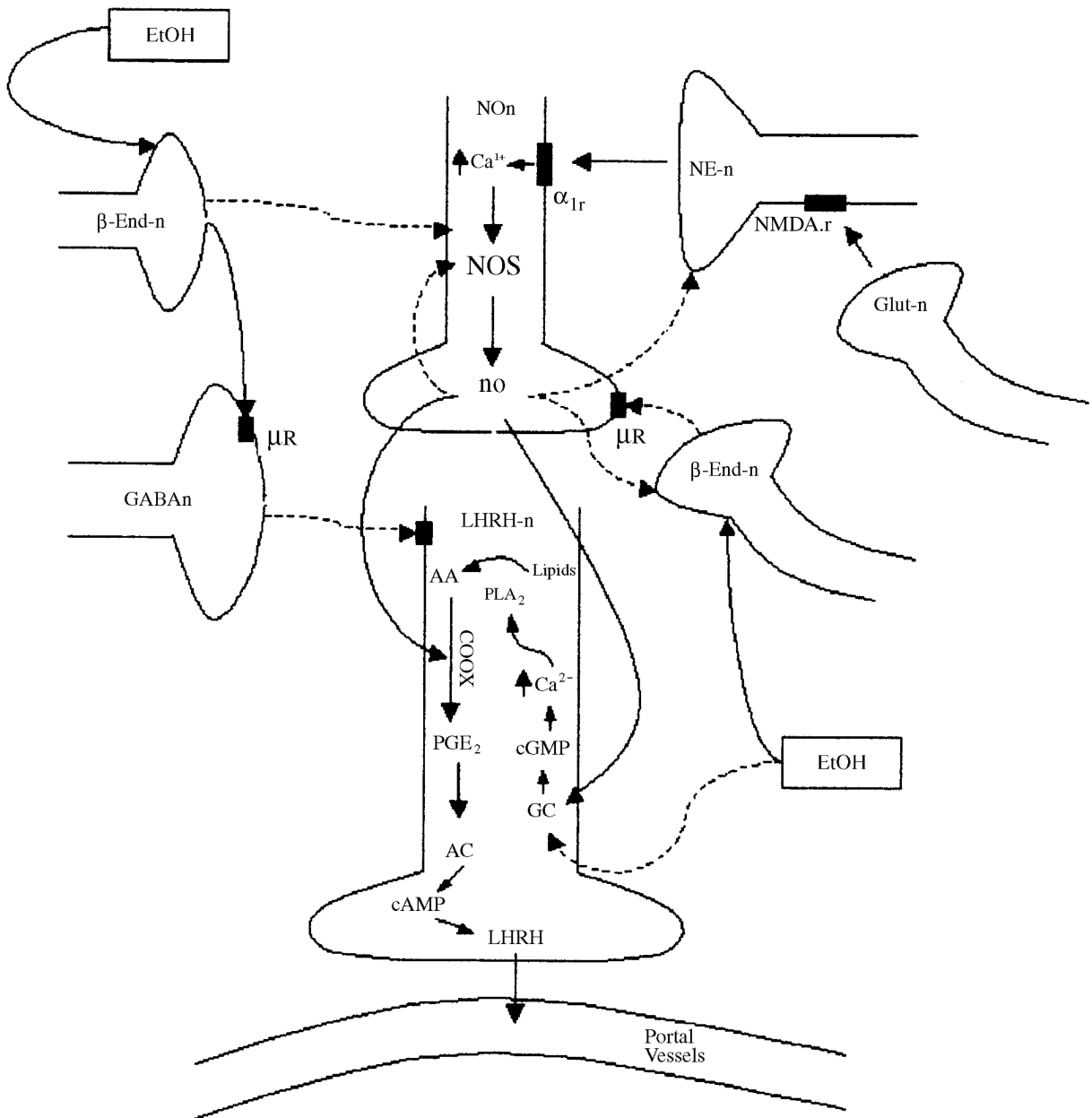


Fig. 1. Diagrammatic representation of the postulated mechanism of action of alcohol (EtOH) to suppress NMDA-stimulated LHRH release. For explanation, see text. β -End, beta-endorphin; μR , μ -opioid receptor; GABA-n, GABA neuron; NO-n, NO-ergic neuron; NE-n, noradrenergic neuron; α_{1r} , α_1 adrenergic receptor; NMDA-r, NMDA receptor; Glut-n, glutamergic neuron; LHRH-n, LHRH neuronal terminal; lipids, membrane phospholipids; PLA₂, phospholipase A₂; GC, guanylate cyclase; AC, adenylate cyclase. Solid arrow indicates stimulation, Dashed arrow indicates inhibition. (From Lomniczi et al., 2000, *Proc. Natl. Acad. Sci. USA*, 97: 2337–2342.)

cleus (Mailleux and Vanderhaeghen, 1992). In order to find out if the CB1 receptors in the hypothalamus are localized on LHRH neurons and terminals,

we performed immunohistochemical studies using an antiserum raised in rabbits against CB1 receptors (anti-rat CB1, working dilution 1:200, kindly do-

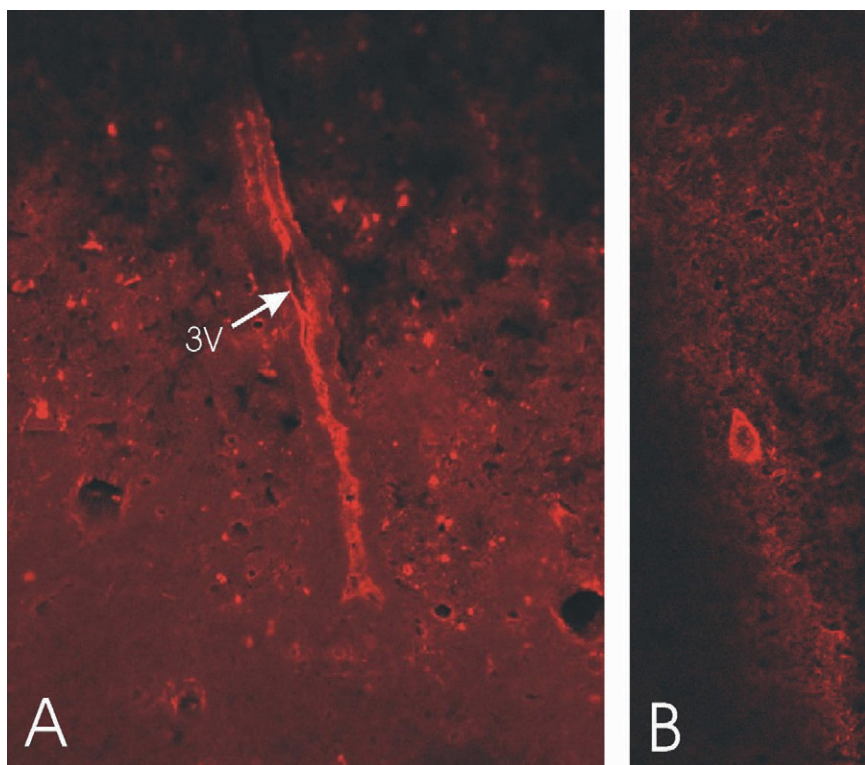


Fig. 2. Transversal sections (20 μ m) of rat hypothalamus immunostained with anti-CB1 receptor (red). (A) 3v: third cerebral ventricle. (B) Detail of a neuron with CB1 receptors.

nated by Dr. K. Mackie, Dept. of Anesthesiology, Univ. of Washington, Seattle, WA, USA). We observed a scattered distribution in the hypothalamus (Fig. 2A and B). Although *ir*-CB1 receptors were distributed in the hypothalamic area where LHRH neurons are found, using double immunohistochemistry techniques, we did not observe colocalization of CB1 receptors with LHRH (data not shown).

These studies suggest that THC most probably is acting on LHRH release by affecting neurotransmitters that are involved in the pathway of LHRH release (Murphy et al., 1998). There is evidence that cannabinoids can inhibit pre-synaptic release of glutamate in rat hippocampus (Shen et al., 1996).

Effect of endogenous cannabinoid, anandamide, on LHRH release

The discovery of specific cannabinoid receptors mediating the effects of marijuana raised the possibility of the existence of endogenous ligands, similar

to the endogenous ligands for opiate receptors, such as beta-endorphin. Endogenous substances that bind to cannabinoid receptors and mimic the action of THC isolated from nervous and peripheral tissues are amides and esters of eicosanoid-like fatty acids. The first such substance isolated from porcine brain by Devane et al., 1992 is N-arachidonyl ethanolamine, named 'anandamide' ('ananda' means 'inner bliss' in Sanskrit) and amide for chemical bonding. Anandamide possesses all the properties of a cannabinoid agonist for CB1 and CB2 receptors (Felder et al., 1993) and as THC causes inhibition of adenylate cyclase. Another endocannabinoid is 2-arachidonylglycerol (Mechoulam et al., 1998). The pathway of anandamide formation is from the hydrolysis of N-arachidonoyl-phosphatidylethanolamine catalyzed by a phospholipase D-like enzyme (Di Marzo et al., 1994). This pathway suggests that anandamide is formed 'on demand' by stimulated cells and accounts for very low levels found in the brain.

As with THC, anandamide was found to be able

to lower plasma LH levels (Wenger et al., 1995). Therefore, we studied the effect of different concentrations of anandamide on LHRH release from MBH in the in vitro system described here. As seen with THC, anandamide (10^{-9} to 10^{-6} M) did not modify significantly the basal release of LHRH from MBH incubated in vitro. When LHRH release was stimulated with NMDA (20 mM) the addition of anandamide together with NMDA inhibited the increase in LHRH release that was evoked by NMDA. This inhibition could be completely reversed by addition of GABA-A antagonist (bicuculline, 10^{-5} M) but not by the opioid antagonist naltrexone (10^{-6} M). Therefore we studied the effect of anandamide on GABA release from MBH in vitro. The addition of anandamide (10^{-9} M) stimulated highly significantly GABA release from MBH. Studies on the effect of anandamide on LH secretion from pituitaries incubated in vitro, showed that only the concentration of 10^{-9} M induced a significant decrease of LH secretion and did not modify the stimulatory response to exogenous LHRH. All these results indicate that anandamide lowers plasma LH mainly by inhibiting the pulsatility of LHRH (since basal release was not modified) and that the pathway is by increased GABA release, a well known inhibitory neurotransmitter that inhibits LHRH release.

In conclusion, all these studies indicate that alcohol and plant derived cannabinoid (THC) as well as endogenous compound (anandamide) have a deleterious effect on reproduction in adult male rats, mainly by lowering plasma LH levels exerting its action mainly at the hypothalamic level by inhibiting LHRH release. Also, they share a common pathway by stimulating inhibitory neurotransmitters such as GABA and consequently inhibiting cyclooxygenase with consequent decrease of PGE_2 release and inhibition of adenylate cyclase with inhibition of cAMP that is necessary for the extrusion of LHRH from its terminals into hypophyseal portal vessels in order to reach the pituitary gland and release LH from gonadotropes.

Acknowledgements

This work was supported by Ministry of Public Health 'Carrillo-Oñativia' grant 2001 and BID 802/OC-AR PICDCT No. 5-6117.

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Glutamatergic regulation of gonadotropin-releasing hormone neurons

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Introduction

The control of the secretory activity of gonadotropin-releasing hormone (GnRH) producing neurons requires complex integration of sensory, seasonal and behavioral cues as well as feedback interactions with the gonadal steroids. Throughout the days of estrus, diestrus I and II of the estrous cycle of the female rat, estradiol exerts an inhibitory effect on the GnRH neurons which release the GnRH peptide at low frequency and low amplitude spikes that maintain basal levels of LH and FSH secretion from the anterior pituitary. Only during proestrus, rising circulating estradiol levels cause an increase in the frequency and amplitude of GnRH release which results in a massive secretion of the luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary gonadotropes which will induce ovulation (Freeman, 1994; Kordon et al., 1994). It had been postulated for a long time that the effects of the gonadal steroids on GnRH release are indirect and that other neurons in the central nervous system convey the stimulatory or inhibitory signals of the steroids to the GnRH neurons. Recently, it was shown, however that certain GnRH neurons do express estrogen receptor- β (Hrabovszky et al., 2001)

which suggests that some effects of estradiol are exerted by directly altering gene transcription in GnRH neurons. These possible direct effects of estradiol are, however, not sufficient to drive the estrous cycle and afferent input from non-GnRH neurons is required for GnRH neurons to release adequate quantities of GnRH into the fenestrated capillaries in the median eminence to induce a preovulatory gonadotropin surge.

Over the past 50 years, numerous studies have implicated almost every neurotransmitter or neuropeptide to have some influence on GnRH release and it is beyond the scope of this article to review all these neuroactive substances. Instead, we will focus on the role of glutamate as a stimulatory neurotransmitter the release of which causes an increase in the secretory activity of GnRH neurons.

Glutamate

Glutamate is the most abundant excitatory neurotransmitter in the brain and its functions and mechanisms of action have been extensively studied in extrahypothalamic sites such as the hippocampus and the Purkinje cells in the cerebellum (for review see Curtis and Johnston, 1974; Monaghan et al., 1989). More recently, several studies have defined the roles of glutamate in the hypothalamus where glutamate is released from nerve terminals in a Ca^{2+} dependent fashion and causes a dramatic increase in Ca^{2+} influx in the postsynaptic neurons (van den Pol, 1991) as well as the generation of strong EP-

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SPs (van den Pol and Trombley, 1993; Belousov and van den Pol, 1997). The view that glutamate is indeed an endogenous neurotransmitter in the hypothalamus is further supported by immunohistochemical studies which clearly show the presence of this amino acid in presynaptic boutons (van den Pol et al., 1990; Decavel and van den Pol, 1992) as has been shown previously in other regions of the brain (Clements et al., 1990). After its release from a presynaptic bouton, glutamate can bind to 2 major classes of postsynaptic receptors, the ion channel forming ionotropic class and the G-protein-coupled metabotropic receptor class. Based upon preferential binding of agonists, the ionotropic class is further divided into NMDA-, AMPA-, and kainate-preferring receptor subunit families which need to assemble with other subunits of the same family to form functional receptor channels. Each family contains several members such as NMDA-R1, -R2A and 2B; Glu-R1, -R2, -R3 and -R4 for the AMPA family and kainate-1 and -2, Glu-R5, -R6 and -R7 for the kainate family (Seeburg, 1993; Hollmann and Heinemann, 1994). Similarly, the metabotropic class of glutamate receptors is divided into three families, depending upon signal transduction mechanisms and agonist potencies. Thus, group I receptors include mGlu-R1 and mGlu-R5 whose activation stimulates phospholipase C, group II receptors include mGlu-R2 and -R3 which inhibit adenylate cyclase and group III which includes mGlu-R4, -R6, -R7 and -R8 which also inhibit cAMP production, however, this group of receptors is activated by the agonist L-AP4 while Group II receptors are activated by trans-MCG-I (Nakanishi, 1994).

Role of glutamate in the regulation of GnRH secretion

Since the mid-seventies it has been known that administration of glutamate can enhance circulating LH levels and that this effect is exerted in the central nervous system since exposure of pituitary slices or dispersed pituitary cells to glutamate or injections of glutamate directly into the pituitary do not alter LH release (Ondo et al., 1976; Schainker and Cicero, 1980; Ondo, 1981; Tal et al., 1983). While these studies offered important first clues about an important participation of glutamate in the regulation

of the brain-pituitary-gonadal axis, no follow-up studies were conducted until renewed interest and new tools became available in the nineties. Since then, numerous studies have been conducted to define the sites and mechanisms of action of glutamate in the regulation of gonadotropin-releasing hormone secretion which have been summarized in several excellent recent reviews (Brann and Mahesh, 1994, 1997). In general, the studies have examined various aspects of an involvement of ionotropic glutamate receptor channels while data on a possible role of the metabotropic receptor class in the control of GnRH neurons are not available. As a brief summary, activation of NMDA, AMPA or kainate receptors by specific agonists or by glutamate enhances GnRH-stimulated LH release in both, male and female animals in a dose-dependent manner. These effects are dependent upon the presence of gonadal steroids since either no or an inhibitory effect of the glutamate agonist NMDA has been observed in ovariectomized animals. Conversely, administration of ionotropic glutamate receptor channel antagonists prevents the preovulatory and the steroid-induced LH surge as well as pulsatile basal LH release which suggests that endogenous glutamate is required for adequate maintenance of basal LH release and preovulatory LH surge generation. This view is supported by push-pull perfusion studies which measured greatly enhanced extracellular glutamate levels in the preoptic area of female ovariectomized and steroid treated rats that paralleled the steroid-induced LH surge which suggests that endogenous glutamate had been released from nerve terminals and activated the GnRH neurons (Jarry et al., 1992).

Localization of glutamate in the hypothalamus

In an effort to determine where in the hypothalamus the glutamate neurons are located that are relevant for the regulation of GnRH release we employed immunohistochemical staining procedures using various mono- and polyclonal antibodies to glutamate (Clements et al., 1990; Petrusz et al., 1990). The results of these studies show that glutamatergic neurons are abundant throughout the hypothalamus, especially in the medial preoptic nucleus and area, median preoptic and preoptic periventricular nucleus and anterodorsal preoptic nucleus, paraventricular

and supraoptic nuclei as well as in the arcuate, dorsomedial and ventromedial nuclei. These results are in agreement with the findings of Ottersen and Storm-Mathisen (1984) who also examined the rat brain and with the data provided by Goldsmith et al. (1994) and Thind and Goldsmith (1995) who examined the brain of cynomolgus monkeys. Dual immunohistochemistry for glutamate and GnRH revealed that many GnRH perikarya and axon terminals in the median eminence are closely apposed by glutamatergic fibers which indicates that glutamatergic input to the GnRH neurons is likely to occur (Eyigor and Jennes, 1996). Interestingly, many of the immunoreactive glutamatergic neurons in the rat (Moore et al., 1999) and in the monkey (Thind and Goldsmith, 1997) also express estrogen receptor- α and, to a lesser extent estrogen receptor- β which suggests that estradiol could directly activate these neurons which in turn could convey the estradiol signal to the GnRH perikarya in the medial septum-diagonal band-preoptic area or to the axon terminals in the median eminence.

Neuron-specific glutamate transporters

Recently, two neuron-specific glutamate transporters have been identified and the cDNA and amino acid sequences have been determined. These transporters have been named VGLUT 1 (vesicular glutamate transporter 1) or BNPI (brain specific Na⁺-dependent inorganic phosphate transporter) (Ni et al., 1994; Bellocchio et al., 2000; Takamori et al., 2000) and VGLUT 2 or DNPI (Na⁺-dependent inorganic phosphate cotransporter) (Aihara et al., 2000; Hisano et al., 2000). These transporters are specific for mediating glutamate uptake by transfected cells while they do not interact with related amino acids such as glutamine or aspartate. Electron microscopic studies have recently confirmed that the glutamate transporters are localized to presynaptic terminals (Bellocchio et al., 1998; Fujiyama et al., 2001). We recently used specific antibodies to both transporters in order to identify more precisely the sites of interactions between the glutamatergic and GnRH-neuronal system. In general, immunoreactive VGLUT 1 and 2 are present in small varicosities or boutons throughout the septum-diagonal band-hypothalamus in extremely dense distribution patterns. It appears that

most neurons in the hypothalamus are juxtaposed by several glutamate transporter containing boutons while in the septum-diagonal band the number of immunoreactive boutons is slightly lower. In general, VGLUT 2 immunoreactive boutons appear slightly larger and more numerous throughout the hypothalamus when compared to VGLUT 1 boutons. Both VGLUT 1 and VGLUT 2 immunoreactive boutons are present in all areas of the ventral diencephalon, however several region-specific differences in the number of boutons containing the transporters are noted. Thus, the medial septum-diagonal band appears to be less densely innervated by VGLUT 1 containing boutons than by VGLUT 2 containing axons as is the mediobasal hypothalamus where the arcuate nucleus contains only relatively sparse VGLUT 1 immunoreactivities and the median eminence which is almost devoid of VGLUT 1 positive structures. In contrast, both the arcuate nucleus and the median eminence are very densely innervated by VGLUT 2 containing boutons which are in many instances juxtaposed to GnRH axon terminals (Fig. 1). Important for the control of GnRH neurons are also the findings that both, immunoreactive VGLUT 1 and VGLUT 2 boutons are found at GnRH perikarya (Fig. 2) which indicates that excitatory glutamatergic input can occur at the GnRH cell bodies as well as at the sites of release of the peptide into the fenestrated capillaries. Based upon the extremely high density of immunoreactive boutons it would be expected that a large number of immunoreactive perikarya should be identified, however, this does not appear to be the case. Only a few immunoreactive cell are visible in the diagonal band, preoptic region and mediobasal hypothalamus which indicates that the transporter protein is either synthesized at a very slow rate or that the proteins are rapidly transported out of the perikaryal region. Future studies are needed to determine which possibility applies.

In order to identify the location of the perikarya that synthesize VGLUT 1 or VGLUT 2 we cloned the cDNA of both transporters and generated cRNA probes for *in situ* hybridization. The results of these studies confirmed that both transporter mRNAs were present in the central nervous system and that the distribution patterns were more or less complementary in that VGLUT 1 mRNA was the predominant transporter mRNA in the cortex and hippocampus

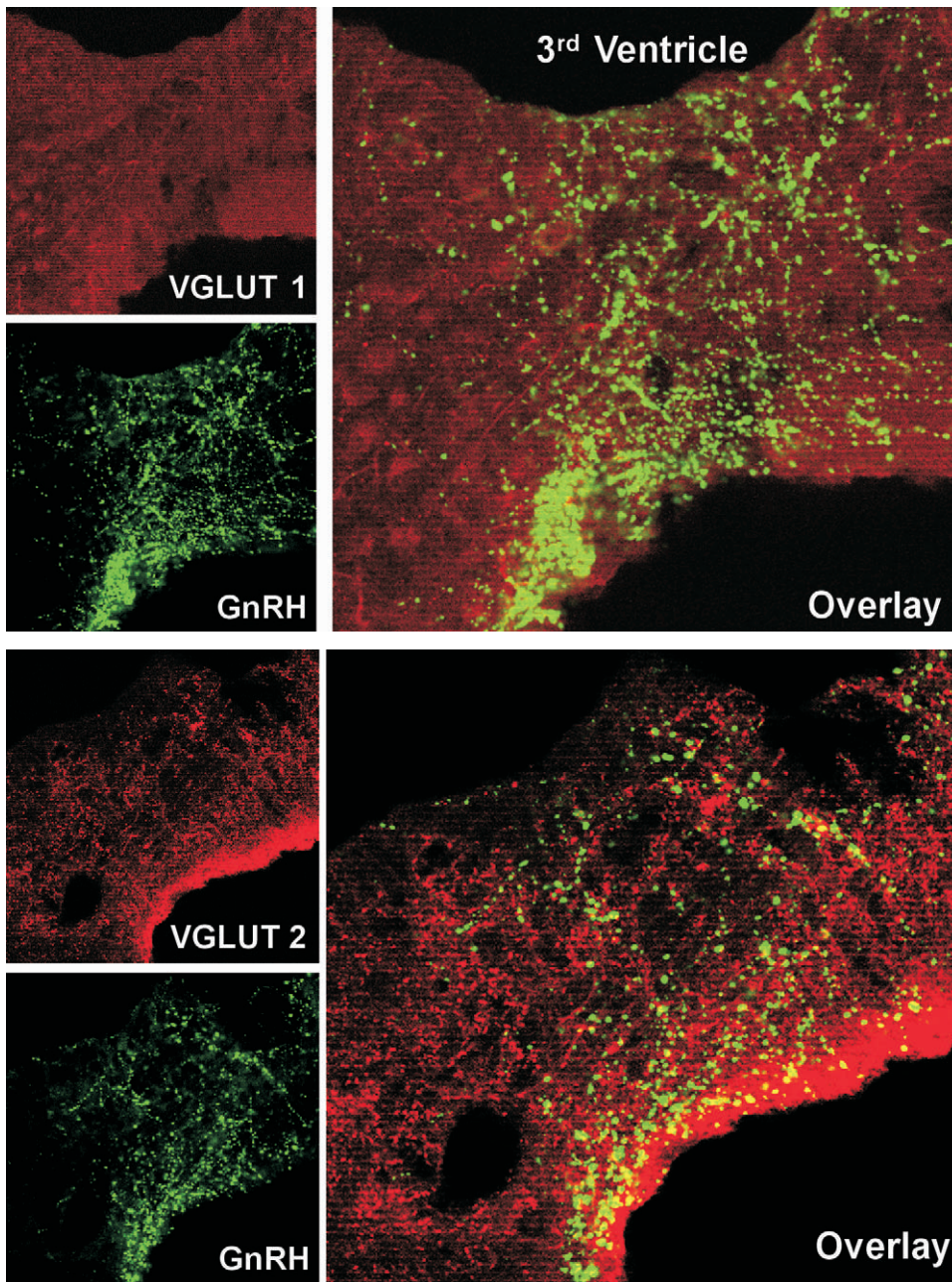


Fig. 1. Immunofluorescence for VGLUT 1 (red) and GnRH (green; top figure) and VGLUT 2 (red) and GnRH (green; bottom figure) and overlays in the rat median eminence showing absence of VGLUT 1 staining while VGLUT 2 is present in numerous boutons many of which are juxtaposed to GnRH axons.

while VGLUT 2 mRNA was much more abundant in the thalamus and hypothalamus. Within the hypothalamus, relatively small amounts of VGLUT 1

mRNA were seen throughout the preoptic region and most of the rostral and central nuclei while VGLUT 2 mRNA was found in slightly larger amounts in

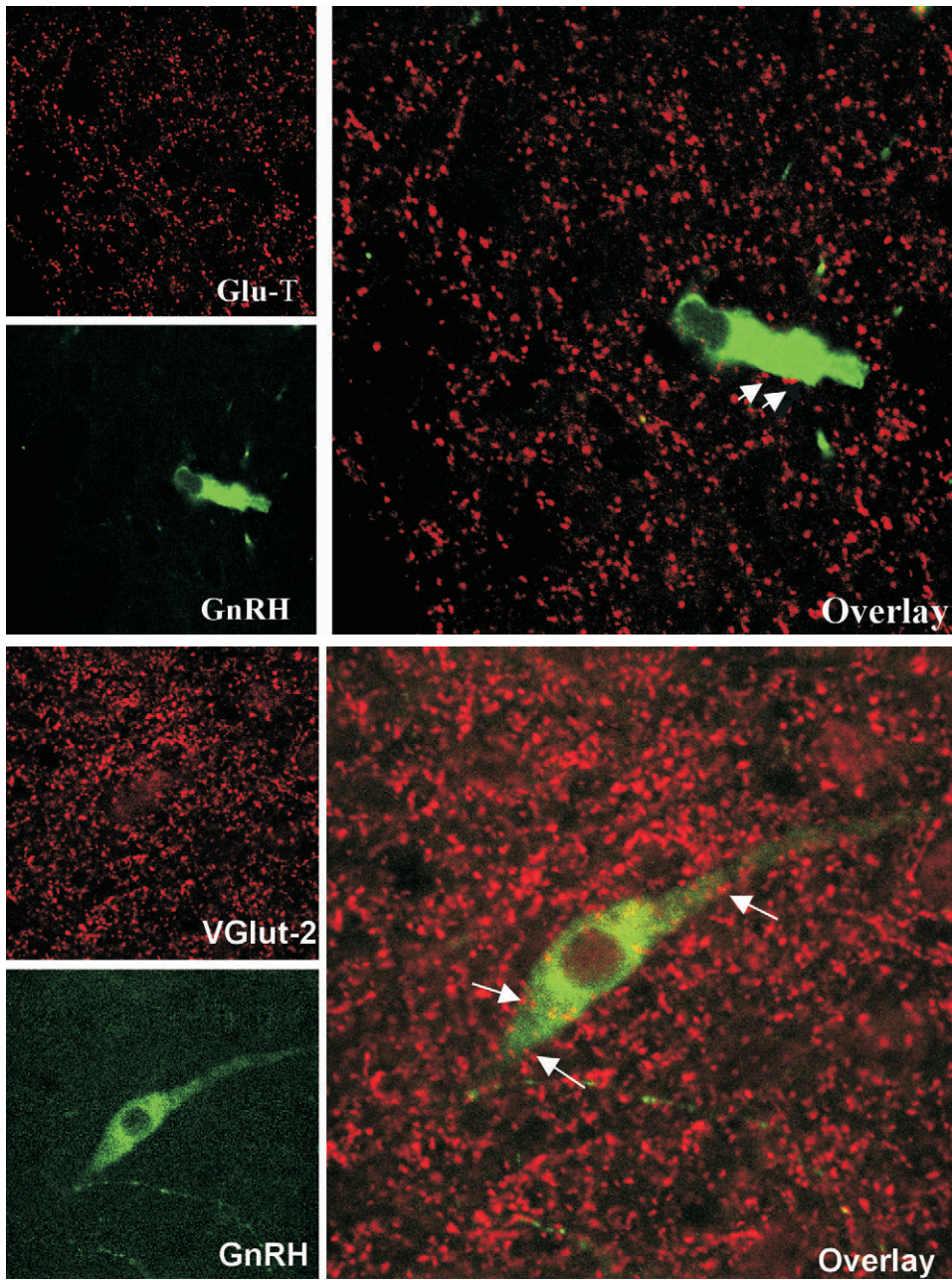


Fig. 2. Immunofluorescence for VGLUT 1 (red) and GnRH (green; top figure) and VGLUT 2 (red) and GnRH (green; bottom figure) and overlays in the ventral septum showing that many VGLUT 1 and VGLUT 2 immunoreactive boutons are juxtaposed to GnRH perikarya (arrows).

the preoptic region as well as the rostral and central nuclei, especially the ventromedial nucleus (Fig. 3). Interestingly, VGLUT 2 mRNA was abundant in the

mammillary complex which did not contain measurable amounts of VGLUT 1 mRNA. The results show that glutamatergic neurons are located in the regions

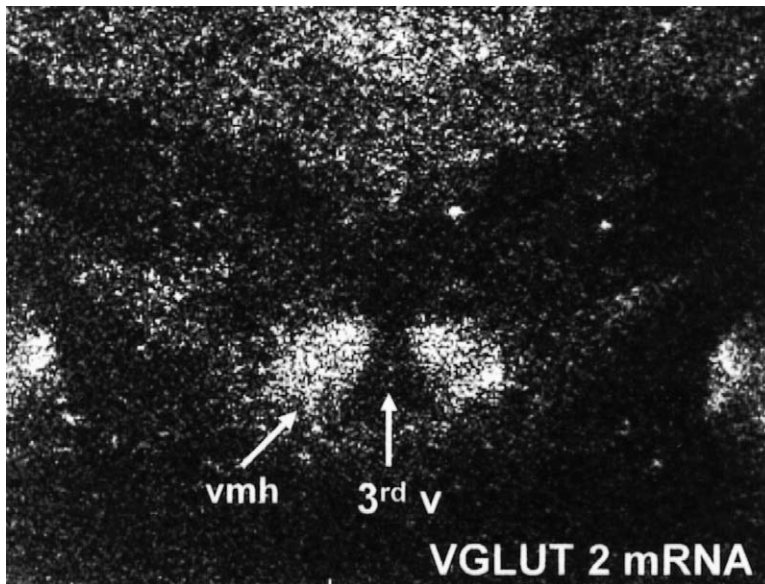


Fig. 3. Autoradiogram after in situ hybridization with ^{35}S -labeled cRNA probes encoding VGLUT 2 in the mediobasal hypothalamus showing abundant VGLUT 2 mRNA in the ventromedial nucleus (vmh).

of the hypothalamus that are relevant for the regulation of GnRH neurons which further strengthens the view that glutamate is intimately involved in the neuroendocrine control of reproduction.

Expression of glutamate receptor subunits in GnRH neurons

Before glutamate can be accepted to be a neurotransmitter that regulates the activity of GnRH neurons, it needs to be established that the GnRH neurons express the relevant postsynaptic glutamate receptors. After the cDNA sequences of the various ionotropic glutamate receptor subunits became available we used in situ hybridization procedures to identify the distribution of each subunit mRNA in the hypothalamus (Eyigor et al., 2001). The results show that all NMDA, AMPA and kainate receptor subunit mRNAs were widely distributed in a subunit-specific distribution pattern and that the NMDA-R1, -R2A and -R2B subunits were the most abundant in the hypothalamus. Interestingly, the extensive overlap in the distribution patterns of the various subunit mRNAs suggests that many neurons can express glutamate receptor channels that belong to different receptor families. Thus, it is likely that these multi-

receptor neurons can integrate the glutamate signal in many different but cell-specific manners.

In order to determine which subunits were expressed in GnRH neurons we applied dual in situ hybridization procedures. The results of these studies showed that the mandatory NMDA-R1 subunit mRNA was apparently absent in most GnRH neurons (Abbud and Smith, 1995; Eyigor and Jennes, 1996) while the NMDA-R2A subunit was detected in 17% of the GnRH neurons. This approach was unable to identify the mRNAs for NMDA-R2B and -R2C in GnRH cells (Eyigor and Jennes, 1996). These results, although unexpected because of the known stimulatory effects of NMDA on GnRH release were supported by the finding that administration of cytotoxic concentrations of the glutamate agonists NMDA or kainic acid into the septum-preoptic region destroys most of the neurons in the affected areas, except for the GnRH neurons (Ebling et al., 1998). These data were interpreted to indicate an absence of NMDA receptors on GnRH neurons. However, more recent studies which employed a more sensitive in situ hybridization or immunohistochemical procedures were able to detect the NMDA-R1 subunit mRNA and protein in the majority of GnRH neurons (Ottem and Petersen, 2000; see also

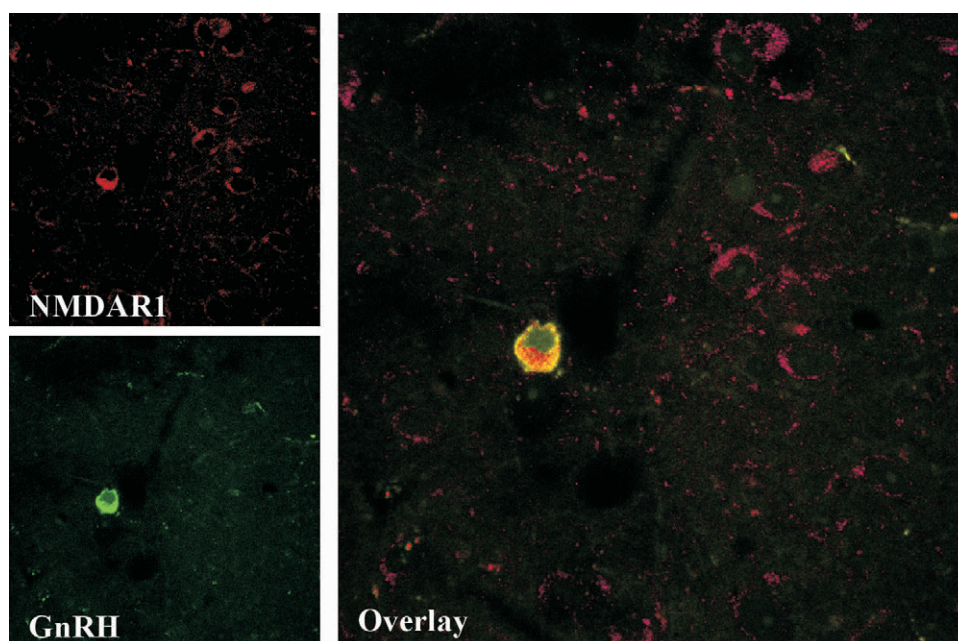


Fig. 4. Immunofluorescence for NMDAR1 receptor subunit (red), GnRH (green) and overlay showing co-localization of this mandatory subunit in a GnRH perikaryon.

Fig. 4) and future studies are needed to show that this subunit assembles with other subunits to form functional channels.

Dual in situ hybridization studies also revealed that less than 5% of the GnRH neurons express the AMPA subunits GluR1–4, however, kainate-2 subunit mRNA was detected in about 50% of the GnRH neurons (Eyigor and Jennes, 1996). Since kainate-2 subunits need to assemble with other kainate-preferring subunits to form functional receptor channels, we examined GnRH neurons for the presence of GluR5, -6 and -7 subunit mRNA and protein. The results show that kainate-2 subunit mRNA containing GnRH neurons also express GluR5 subunit mRNA while GluR6 and -7 mRNAs were not detected. These results have been confirmed with multiple immunohistochemical stainings indicating that the kainate receptor subunit mRNAs are translated into protein and it can be expected that in GnRH neurons, kainate 2 subunits assemble with GluR5 subunits to form functioning receptor channels (Eyigor and Jennes, 2000; see also Fig. 5).

These receptors may be important for the control of the preovulatory or steroid-induced LH surge

since most kainate receptor expressing GnRH neurons belong to the subpopulation of GnRH neurons that synthesizes the transcription factor *fos* during the surge (Eyigor and Jennes, 2000). It is thought that this subpopulation of GnRH neurons is functionally different from the *fos*-negative GnRH neurons and that the presence of *fos* in these cells is an indicator of cellular activation (for review see Hoffman et al., 1993). Thus, GnRH neurons that express *fos* have a higher GnRH mRNA content than *fos*-negative GnRH neurons (Wang et al., 1995) and the number of *fos*-positive GnRH neurons parallels magnitude of the LH surge (Attardi et al., 1997). The induction of *fos* in GnRH neurons during the LH surge suggests that this transcription factor may control the replenishing of GnRH peptide in depleted neurons since an activation protein (AP)-1 site is present in the GnRH promoter (Bond et al., 1989) to which a *fos* heterodimer could bind.

Recently, a transgenic mouse that expresses green fluorescent protein under the control of the GnRH promoter has been generated and used to determine the electrophysiological properties of identified GnRH neurons in slice preparations (Spergel et al.,

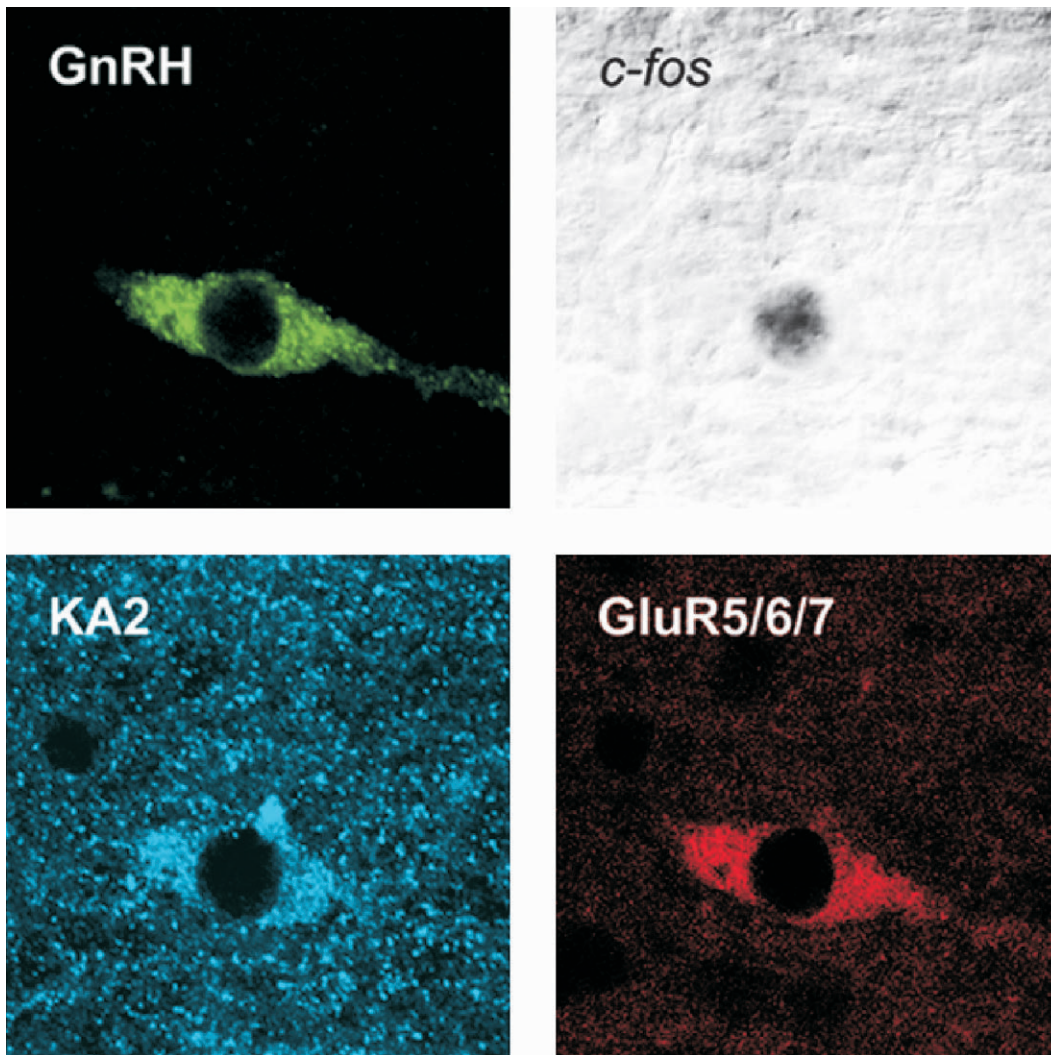


Fig. 5. Immunohistochemical quadruple staining for GnRH (green), GluR5, -6, -7 (red), kainate-2 (blue) and *fos* (black) showing an 'activated' GnRH neuron in the ventral septum that co-expresses two kainate receptor subunits.

1999). These studies clearly showed that functional glutamate receptor channels are present in the plasma membrane of the GnRH neurons and, based upon the effects of antagonists it appears that in the mouse most of the responses to glutamate were apparently mediated by AMPA receptors and less by NMDA receptors. Since these electrophysiological data obtained in the mouse are very different from the results obtained in the rat with in situ hybridization, it remains to be determined if there is a significant species difference in the expression patterns of gluta-

mate receptor subunits in GnRH neurons or if other explanations, such as differences in the sensitivity of the detection methods apply.

Conclusion

The data presented in this article support the view that glutamate is an important neurotransmitter in the control of GnRH neuronal activity. Based upon the morphological data, it is suggested that certain glutamatergic neurons express estrogen receptor- α and

these neurons are present in the critical regions of the hypothalamus that are known to be involved in the neuroendocrine control of reproduction, such as the preoptic region, ventromedial nucleus and arcuate nucleus. Furthermore, glutamatergic axons appear to contact GnRH perikarya in the medial septum–diagonal band–rostral preoptic region as well as GnRH containing axons in the median eminence. Since GnRH neurons express two kainate preferring receptor subunits, the GluR5 and kainate 2 subunits, it is likely that some of the effects of glutamate on GnRH neurons are mediated by kainate receptor channels. Recent evidence also suggests that NMDA receptors may be involved since many GnRH neurons express the NMDAR1 subunit while at present the identity of the companion subunits is not clear.

Acknowledgements

This work was supported by NIH grants AG17164 and MH59890 (LJ).

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Gonadotropin-releasing hormone (GnRH) neurons: gene expression and neuroanatomical studies

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Introduction

Gonadotropin-releasing hormone (GnRH) neurons are the key cells regulating reproductive function in all vertebrate organisms. In mammals, birds, reptiles and amphibians, the GnRH-1 decapeptide is released in a pulsatile manner from neuroterminals in the median eminence into the portal capillary vasculature, where it binds to receptors located on pituitary gonadotropes to regulate the synthesis and secretion of the gonadotropins. In fish, GnRH-1 is released directly into the anterior pituitary gland where it stimulates gonadotropin release. The gonadotropins (luteinizing hormone, LH, and follicle-stimulating hormone, FSH) are secreted into the peripheral circulation where they act at the gonads to regulate steroidogenesis, spermatogenesis in males, and folliculogenesis and oogenesis in females.

Although each level of the hypothalamic–pituitary–gonadal axis is critical for normal reproductive function, the GnRH neurons are the *primary* regulator of this axis. In the absence of GnRH neurons, as in the genetic mutations of Kallmann's syndrome in humans, or the hypogonadal (hpg) mouse, reproductive development never occurs spontaneously

(Kallmann et al., 1944; Gibson et al., 1997). GnRH neurons in turn are regulated by inputs from other neurotransmitters, neuropeptides, neurotrophic factors, as well as by feedback from gonadal steroid hormones. Thus, GnRH neurons serve to integrate crucial information about the internal and external environment, thereby coordinating the timing of reproductive physiology with the proper behavioral, environmental and homeostatic cues.

The goal of this chapter is to review the work from my laboratory and others on GnRH-1 gene expression and neuroanatomy. In the first section, studies on GnRH gene expression have been undertaken in order to determine the relationship between GnRH biosynthesis and secretion. GnRH release varies depending upon a number of physiological stimuli, including the time of year in seasonal breeders, the time of day (i.e., circadian or diurnal fluctuations), the stage of the cycle in females (e.g., menstrual or estrous cycles), as well as the age of the animal (e.g., prepubertal vs. post-pubertal, and adult vs. aging animals). It seems reasonable to predict that GnRH gene expression is affected in a similar manner as GnRH release. However, as I will discuss below, in many cases, alterations in GnRH gene expression do not necessarily parallel these alterations in GnRH release, or the former may be much less robust than the latter. Moreover, the mechanisms for the regulation of GnRH mRNA levels can vary depending upon the experimental protocol or the physiological condition of the organism.

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The second part of this chapter will focus on the neuroanatomical regulation of GnRH-1 neurons. Many laboratories have reported in diverse species that the number of GnRH-1 neurons does not vary substantially during postnatal life, even during those periods when it might be predicted that such changes may occur, including puberty or reproductive senescence (Hoffman and Finch, 1986; Witkin, 1986; Wray and Hoffman, 1986; Rubin and Bridges, 1989; Gore et al., 1996). Moreover, numbers of GnRH cells are similar among males and females of a species (Wray and Hoffman, 1986). Thus, the regulation of GnRH neurons probably occurs primarily by changes in inputs to GnRH cells from other neurons in the brain. I have been using two model systems for studying this phenomenon: the neurotransmitter glutamate, acting through the N-methyl-D-aspartate (NMDA) receptor, and the neurotrophic factor insulin-like growth factor-I (IGF-I); this chapter will focus on these two substances, with an emphasis on NMDA receptor studies. Nevertheless, many other neurotransmitters and neurotrophic factors are extremely important in the regulation of GnRH cellular functions, and it is necessary to take these other factors into consideration in understanding the 'big picture' of the GnRH neurosecretory system.

I would like to add a brief note on the nomenclature used in this review paper. The GnRH neurons under investigation in studies in my laboratory are the hypophysiotropic, or GnRH-1 population of neurons (Fernald and White, 1999; Gore, 2002). These cells are found predominantly in the preoptic area (POA), organum vasculosum of the lamina terminalis (OVLT), septum and anterior hypothalamus. GnRH-1 neurons project a neuroterminal to the median eminence, where the GnRH decapeptide is released. Two other populations of GnRH neurons have been found in the brain: the GnRH-2 cells, located predominantly in the midbrain, hindbrain and/or non-reproductive hypothalamic regions, and the GnRH-3 cells, associated with olfactory regions and the terminal nerve (Fernald and White, 1999; Parhar et al., 2000). The functions and anatomy of the GnRH-2 and GnRH-3 populations are probably quite different from those of the GnRH-1 cells, and therefore this chapter will focus only on GnRH-1 neurons.

Regulation of GnRH gene expression

General observations

GnRH mRNA levels vary during development, across reproductive cycles of females, and are regulated by neurotransmitters, neurotrophic factors and steroid hormone feedback. Several general conclusions on the regulation of GnRH gene expression can be drawn from the literature. First, changes in GnRH mRNA levels in vivo commonly occur in response to experimental manipulations (e.g. following gonadectomy or steroid hormone replacement, in response to pharmacological agents) and during reproductive development. With the exception of developmental changes, most alterations in GnRH mRNA levels are generally not very large (i.e., on the order of 20–50%) and thus they are relatively small in comparison to concomitant changes in GnRH release. Second, much of the regulation of GnRH mRNA levels appears to occur at a post-transcriptional level (Gore and Roberts, 1997). Those studies that have measured GnRH gene transcription together with GnRH mRNA levels in vivo often report that GnRH mRNA levels change independently of GnRH gene transcription. Third, GnRH mRNA levels can be regulated quite rapidly in the animal, as early as fifteen minutes after treatment with a pharmacological agent such as NMDA (Petersen et al., 1991; Liaw and Barraclough, 1993; Gore and Roberts, 1994). Such a rapid response is consistent with a post-transcriptional change in GnRH mRNA stability.

Developmental changes in GnRH mRNA levels

GnRH release changes profoundly during reproductive development. During the late gestational and early postnatal periods, GnRH release is elevated, and may be disorganized (Donovan et al., 1975; Doecke et al., 1978; Ramaley, 1979; Plant, 1988; Gore, 2000). Then, a period of low GnRH release ensues, often called the 'prepubertal hiatus'. Although the mechanisms for the hiatus are not completely understood, this relative quiescence of the GnRH neurosecretory system may be attributable to inhibitory inputs from other neurons that suppress GnRH release. Following this quiescent period, GnRH release

begins to increase at the onset of puberty, and during the progression of puberty, the amplitude and frequency of GnRH pulses continue to increase until adult reproductive function is attained (Urbanski and Ojeda, 1985; Plant, 1988; Watanabe and Terasawa, 1989; Gore, 2000; Sisk et al., 2001). Because the physiological demand for GnRH varies depending upon the life stage of the organism, it is of interest to determine how GnRH biosynthesis, measured by levels of GnRH gene expression, changes during reproductive maturation. Studies in rodents have consistently shown that GnRH mRNA levels increase during the pubertal process, and this may play a role in regulating the increasing GnRH peptide levels during puberty.

The relationship between GnRH release and gene expression has been studied in my laboratory by measuring changes in GnRH gene expression in developing male and female C57bl/6 mice, beginning at embryonic day (E) 16 through postnatal day (P) 60 (Gore et al., 1999). Using the highly quantitative RNase protection assay, we reported that GnRH mRNA levels increase gradually and significantly, by approximately 15-fold during this developmental period, peaking on P60 in females, and P40 in males (Fig. 1; Gore et al., 1999). Most of the changes in GnRH mRNA levels are associated with the pubertal period of development (i.e., beginning in the third to fourth week of postnatal life), whereas few changes in GnRH mRNA levels occur earlier in development, i.e., during the first three postnatal weeks of life (Adams et al., 1999; Gore et al., 1999). Most other reports support the concept that GnRH mRNA levels increase during the pubertal period in rats, mice and hamsters (Jakubowski et al., 1991; Dutlow et al., 1992; Gore et al., 1996, 1999; Parfitt et al., 1999). Our laboratory also demonstrated an association between the increase in GnRH mRNA levels during pubertal development in female rats and the timing of vaginal opening, an index of reproductive maturation (Gore et al., 1996), and this suggests a physiological relevance to the pubertal increase in GnRH mRNA levels. In addition, to my knowledge, this developmental increase in GnRH mRNA levels is the most dramatic such change identified in vivo.

Mechanisms for the increase in GnRH mRNA during development

In order to determine the mechanism for the increase in GnRH mRNA levels during maturation, we measured GnRH primary transcript levels in the same animals in which we had measured GnRH mRNA (Fig. 1; Gore et al., 1999). GnRH primary transcript levels, which are measured by RNase protection assay using a probe containing the intron B-exon 2-intron C junction of the proGnRH gene, representing the unprocessed GnRH RNA precursor (Jakubowski and Roberts, 1994), are used as an index of GnRH gene transcription in vivo (Yeo et al., 1996). Overall, developmental changes in GnRH primary transcript are quite different from those of GnRH mRNA, and moreover, are sexually dimorphic during the neonatal period. In males, GnRH primary transcript levels are low until P5; they then undergo a 4-fold increase between P5 and P7. Levels continue to increase through P15, at which time they reach a plateau that is maintained through adulthood (Gore et al., 1999). In females, GnRH primary transcript levels are high at E16, decrease to a nadir at P5, and then increase 5-fold from P5 to P7, at which time adult levels are attained (Gore et al., 1999). These results are similar to those obtained using a transgenic mouse model which undergoes large increases in GnRH gene transcription from the first to second postnatal week of life (Wolfe et al., 1996). Thus, the increase in GnRH gene transcription occurs much earlier in development than the increase in GnRH mRNA levels. Taken together, these findings support the concept that the pubertal increase in GnRH mRNA levels is regulated to a large extent by a post-transcriptional mechanism, as GnRH gene transcription has already reached adult levels at that time.

Regulation of GnRH gene expression by the NMDA receptor

The excitatory amino acid glutamate, acting through both N-methyl-D-aspartate (NMDA) and non-NMDA receptors, stimulates GnRH and LH release (Brann, 1995; Gore, 2000, 2001). Glutamate receptors have also been shown to regulate GnRH gene expression. Effects of NMDA receptor activation on GnRH mRNA levels in rats have been reported by

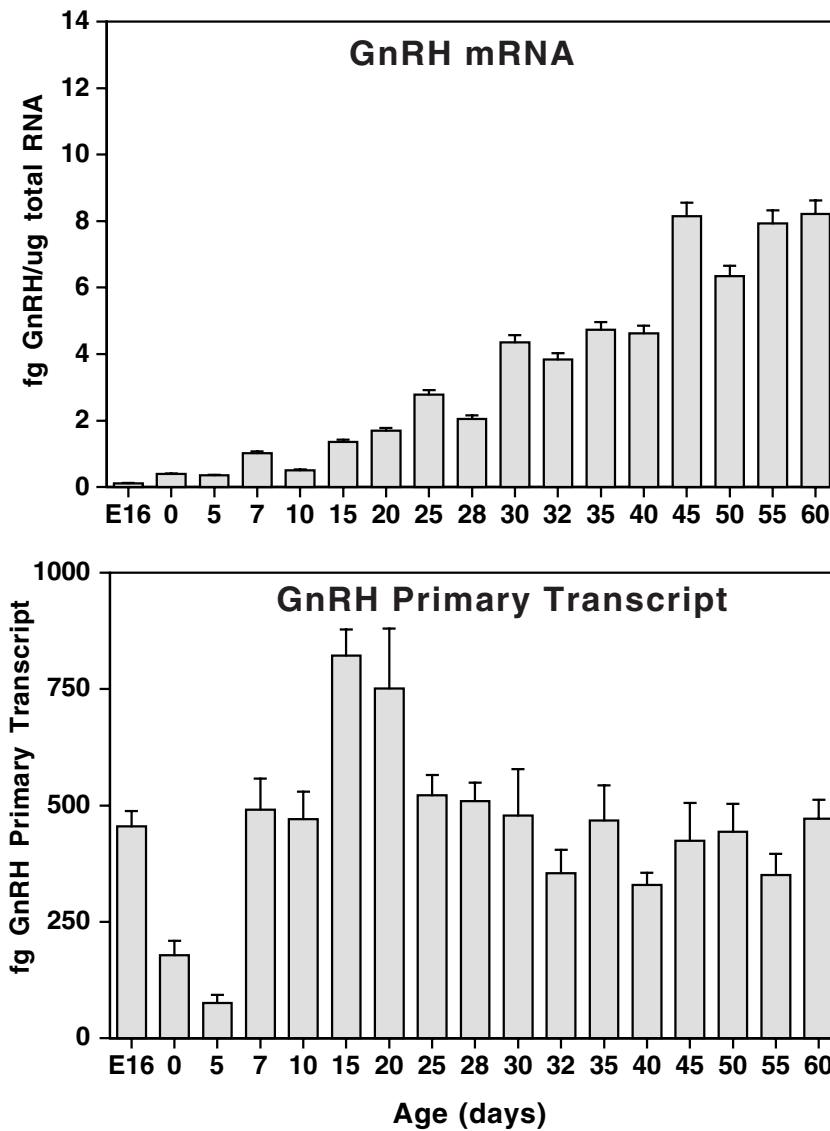


Fig. 1. Changes in GnRH mRNA (top) and primary transcript (bottom) during development in female mice. GnRH mRNA levels, normalized to total μg of RNA in the POA, increase gradually and significantly during postnatal development. GnRH primary transcript levels in the POA are high at E16, decrease to a nadir at P5, then increase again by P7, at which time levels are maintained through adulthood. Abbreviations: E: embryonic day; P: postnatal day; POA: preoptic area. Modified from Gore et al., 1999.

several laboratories, and the results have consistently demonstrated stimulatory effects of NMDA receptor activation on GnRH mRNA levels. In young adult male and female rats, GnRH mRNA levels are significantly elevated 15 minutes to one hour after treatment with NMDA agonists (Petersen et al., 1991; Liaw and Barraclough, 1993; Gore and

Roberts, 1994; Gore et al., 2000a). Activation of non-NMDA (kainate) receptors also rapidly elevates GnRH mRNA levels in male rats (Gore and Roberts, 1994).

Because NMDA receptor activation causes such a rapid increase in GnRH mRNA levels, we speculated that this may be due to a post-transcriptional effect

(Gore and Roberts, 1994). The mechanism for the stimulation of GnRH mRNA levels was examined by measuring steady-state GnRH primary transcript levels as an index of GnRH gene transcription (Yeo et al., 1996). Following NMDA or kainate receptor activation, GnRH mRNA levels are elevated, while GnRH primary transcript levels remain unchanged. Thus, the increase in GnRH mRNA levels occurs by a post-transcriptional mechanism, probably mRNA stability, as GnRH gene transcription is not affected by activation of glutamate receptors.

NMDA receptor regulation of GnRH gene expression during development and puberty

Although an increase in pulsatile GnRH release is the rate-limiting step for the onset and progression of puberty, this is subject to regulation by afferent inputs. Glutamate is one of the primary excitatory neurotransmitters implicated in playing such a role in puberty as well as during adulthood, and the NMDA receptor has been most widely studied in mediating this effect. A role for excitatory glutamatergic input in the stimulation of puberty has been suggested by reports demonstrating that activation of the NMDA receptor advances the timing of puberty, and that blockade of NMDA receptors delays the timing of puberty (Gay and Plant, 1987; Urbanski and Ojeda, 1990; Smyth and Wilkinson, 1994; Gore et al., 1996). Treatment of prepubertal animals with NMDA receptor agonists stimulates GnRH and LH release (Gay and Plant, 1987; Urbanski and Ojeda, 1990; Smyth and Wilkinson, 1994; Gore et al., 1996), and the sensitivity of GnRH neurons to NMDA receptor activation peaks during the peripubertal period (Cicero et al., 1988; Smyth and Wilkinson, 1994).

Effects of NMDA receptor activation on GnRH gene expression have been studied from a developmental perspective. Neonatal male and female rats are not very sensitive to treatment with an NMDA receptor agonist or antagonist, which exert only small effects on GnRH gene expression (Adams et al., 1999). Nevertheless, those effects that are observed are consistent with a stimulatory effect of NMDA, and inhibitory effect of MK-801, on GnRH mRNA levels (Adams et al., 1999), similar to effects in mature animals.

We investigated the relevance of the increase in GnRH mRNA to the timing of puberty. To do this, we induced precocious puberty by activation of the NMDA receptor. As discussed above, we had found that GnRH mRNA levels increase during the pubertal period, and that this is associated with the timing of vaginal opening in female rats (Gore et al., 1996). In subsequent studies, we administered an NMDA receptor agonist, N-methyl-D,L-aspartate (NMA), to prepubertal female rats. This causes an advancement in the timing of the onset of puberty (vaginal opening and first diestrus) by approximately five days in our rats, similar to other studies (Gay and Plant, 1987; Urbanski and Ojeda, 1990; Smyth and Wilkinson, 1994; Gore et al., 1996). NMA treatment causes an increase in GnRH mRNA levels coincidentally with the day of vaginal opening (Gore et al., 1996). We also observed that GnRH primary transcript levels are unaltered by this NMA treatment regime, indicating that the increase in GnRH mRNA levels in rats undergoing precocious puberty is due to a post-transcriptional mechanism (Gore et al., 1996). These data support the hypothesis that an increase in GnRH mRNA levels is associated with, and may be causally related to, the process underlying pubertal maturation and the attainment of adult reproductive function. In addition, the change in GnRH mRNA levels is probably due to a post-transcriptional event such as an enhancement of GnRH mRNA stability.

Regulation of GnRH gene expression by ovarian steroid hormones

The regulation of GnRH gene expression by sex steroid hormones has been intensively studied (Table 1; reviewed in Pfaff, 1986; Sagrillo et al., 1996; Gore and Roberts, 1997). Nevertheless, even the most seemingly straightforward experimental models have yielded considerably different results, depending upon the age, hormonal treatment, and method of detection of the GnRH mRNA. Several generalities may be drawn from the literature on intact female rats. First, most reports agree that GnRH gene expression varies across the estrous cycle (Pfaff, 1986; Sagrillo et al., 1996; Gore and Roberts, 1997; Gore, 2002). Second, a peak in GnRH mRNA levels occurs shortly before or during the preovulatory GnRH/LH surge (Table 1; Zoeller and Young, 1988; Park et

TABLE 1

GnRH gene expression: regulation by estrogen in female rats^a

A. Intact female rats (estrous cycle and GnRH/LH surge)

- GnRH mRNA levels peak at 1200 h on proestrus (ISH) (Porkka-Heiskanen et al., 1994)
- GnRH mRNA levels peak at 1500 h on proestrus, and change during the estrous cycle (RPA) (Gore and Roberts, 1995; Gore et al., 2000b)
- GnRH mRNA levels peak at 1800 h on proestrus (ISH) (Park et al., 1990)
- GnRH mRNA levels peak at 1900 h on proestrus, and vary across the estrous cycle (ISH) (Zoeller and Young, 1988)
- Two peaks in GnRH mRNA, at 1100 h on proestrus and estrus (RT-PCR) (Suzuki et al., 1995)
- No change in GnRH mRNA between 1200 and 1800 h on proestrus (ISH) (Marks et al., 1993)
- No differences in GnRH mRNA across estrous cycle (ISH) (Malik et al., 1991)

B. Effects of estrogen in OVX rats

Stimulatory effects of estrogen:

- E2 (1 d) increases in GnRH mRNA (ISH) (Rosie et al., 1990)
- E2 (1 or 4 d) increases GnRH mRNA (RPA) (Roberts et al., 1989)
- E2 (2 d) increases GnRH mRNA (ISH) (Park et al., 1990)
- E2 (2 d) increases GnRH mRNA with peak at 1200 h (ISH) (Petersen et al., 1995, 1996)
- E2 (1–2 d) increases GnRH mRNA in immature rats (RPA) (Attardi et al., 1997)
- E2 (7 d) increases GnRH mRNA (ISH) (Pfaff, 1986; Rothfeld et al., 1989)

Inhibitory effects of estrogen:

- E2 (2 d treatment) decreases GnRH mRNA (ISH) (Zoeller et al., 1988)
- E2 (14 d) decreases GnRH mRNA (ISH) (Toranzo et al., 1989)
- E2 (18 d) decreases GnRH mRNA in slice explant cultures (ISH) (Wray et al., 1989)

No effects of estrogen:

- No change in GnRH mRNA following E2 (2 d) (RPA) (Gore and Roberts, 1995)
- No change in GnRH mRNA following E2 (2d) (ISH) (Marks et al., 1994)

C. Effects of OVX on GnRH gene expression in female rats

- OVX (2 d) decreases GnRH mRNA levels in immature rats (Slot-blot) (Kim et al., 1989)
- OVX (2–24 d) decreases GnRH mRNA levels (RPA) (Roberts et al., 1989)
- OVX (3 d) increases GnRH mRNA levels (ISH) (Li et al., 1995)
- OVX (14 d) increases GnRH mRNA levels (ISH) (Toranzo et al., 1989)
- OVX (14 d) has no effect on GnRH mRNA levels (ISH) (Kelly et al., 1989)

^a The technique used to detect GnRH mRNA is indicated in parentheses.

Abbreviations: RPA: RNase protection assay; ISH: In situ hybridization; NB: Northern blot; E2: estrogen; OVX: ovariectomy; d: days.

al., 1990; Porkka-Heiskanen et al., 1994; Gore and Roberts, 1995; Suzuki et al., 1995). Third, these changes in GnRH mRNA levels on proestrus tend to be relatively small (e.g., approximately 30–50%); this is of much lower magnitude than changes in preovulatory GnRH release (Sarkar and Fink, 1980; Levine and Ramirez, 1982; Park and Ramirez, 1989).

I had reported that GnRH mRNA levels fluctuate during the estrous cycle, with a peak occurring on diestrus 2, the day before the preovulatory GnRH/LH surge, and a second peak occurring on proestrus at 1500 h, shortly before the GnRH/LH surge (Gore and Roberts, 1995; Gore et al., 2000b). The mechanism for these increases in GnRH mRNA levels was examined by measuring levels of GnRH

primary transcript in these same animals. GnRH primary transcript increases in parallel with GnRH mRNA at 1500 h on proestrus; this suggests that the increase in GnRH mRNA levels at this time is due at least in part to an activation of GnRH gene transcription (Gore and Roberts, 1995). I believe that this peak in GnRH gene expression (both gene transcription and mRNA levels) occurring prior to or during the preovulatory GnRH/LH surge may prepare the organism for the large preovulatory increase in GnRH release, or may help to replenish stores of GnRH peptide that may be depleted during the surge. Further support for this concept is provided by studies demonstrating that blockade of the GnRH/LH surge with pentobarbital prevents the increase in

GnRH mRNA on proestrus from occurring (Park et al., 1990; Gore and Roberts, 1995). Thus, there is a temporal relationship between GnRH biosynthesis and the preovulatory GnRH/LH surge.

GnRH gene regulation by ovariectomy (OVX) and estrogen replacement in female rats

The regulation of GnRH mRNA levels by OVX and steroid treatment has proven to be a very controversial field (Table 1). For example, stimulatory, inhibitory or no effects of OVX on GnRH gene expression have been reported. Thus, one group found no effect of OVX on GnRH mRNA levels (Kelly et al., 1989), two laboratories demonstrated OVX-induced increases (Toranzo et al., 1989; Li et al., 1995), and two groups reported OVX-induced decreases in GnRH mRNA (Kim et al., 1989; Roberts et al., 1989). Effects of estrogen on GnRH mRNA levels in OVX female rats have also been studied, and again, very different results have been obtained, although these may be more generalizable (Table 1). Some of the differences between experiments may be due to different experimental protocols for estrogen replacement (length of treatment, time after OVX at which treatment was initiated, dose, presence or absence of progesterone, age of the animal, time of day of the experiment). Nevertheless, several general trends emerge. First, short-term treatment with estrogen (e.g., 1–2 days) usually results in stimulatory effects on GnRH mRNA levels (Roberts et al., 1989; Park et al., 1990; Rosie et al., 1990; Petersen et al., 1995, 1996; Attardi et al., 1997). Second, studies using long-term treatment with estrogen (14–18 days) generally report negative effects on GnRH mRNA levels (Toranzo et al., 1989; Wray et al., 1989). Third, progesterone generally has not been shown to have consistent or strong effects on GnRH gene expression (Marks et al., 1994; Gore and Roberts, 1995; Petersen et al., 1995). Fourth, and perhaps most importantly, these effects of estrogen on GnRH gene expression are relatively small, generally in the range of 20–50%, as is also the case in intact female rats during the preovulatory GnRH/LH surge (see above). Therefore the GnRH/LH surge, either natural or steroid-induced does not require a massive induction of the GnRH gene either to accumulate or replenish stores of GnRH decapeptide (Gore, 2002).

Reproductive senescence

The role of hypothalamic GnRH neurons in the timing and progression of reproductive senescence is not well-understood. Most studies on menopause in women have focused on ovarian changes, particularly the dramatic follicular atresia that results in a precipitous decline in estrogen levels. However, studies on animals are making it increasingly clear that changes in other levels of the hypothalamic–pituitary–gonadal axis, including hypothalamic GnRH neurons, may also play a role in the process of reproductive senescence.

Female rodents, unlike primates, do not experience a phenomenon equivalent to menopause. Because rodents experience estrous cycles but not menstrual cycles, their reproductive senescence is better termed ‘estropause’ as opposed to the primate equivalent of menopause. As rodents age, their estrous cycles become more and more irregular, and eventually, the rodents enter a stage of acyclicity termed persistent estrus. However, this transition to acyclicity occurs independently of ovarian follicular atresia, as the ovaries of old, acyclic rodents remain capable of resuming ovulation in response to external stimuli, and contain functional follicles (Huang and Meites, 1975; Lu et al., 1977). Therefore, ‘estropause’ in rodents must be due to alterations in hypothalamic and/or pituitary drive to the ovaries.

Changes in GnRH gene expression during reproductive aging

Although the number of GnRH neurons does not change substantially during reproductive aging (Hoffman and Finch, 1986; Rubin and Bridges, 1989), GnRH neurons undergo several functional changes in aging rats, prior to reproductive failure. First, pulsatile GnRH and luteinizing hormone release change during aging (Rubin and Bridges, 1989; Hwang et al., 1990; Sortino et al., 1996; Zuo et al., 1996). Second, the preovulatory GnRH/LH surge is significantly attenuated in middle-aged compared to young rats (Steger et al., 1980; Wise, 1984; Nelson et al., 1992; Rubin and King, 1994). Third, the expression of the immediate early gene Fos, a marker of gene activation, is lower in GnRH neurons of middle-aged compared to young rats during the pre-

ovulatory GnRH/LH surge (Lee et al., 1990; Lloyd et al., 1994; Rubin et al., 1994). These findings are all consistent with an age-related alteration at the level of the GnRH neuron itself that may play a role in 'estropause' in female rodents.

GnRH gene expression changes during reproductive senescence in rats. Studies from our laboratory on intact female rats have demonstrated an age-related increase in GnRH mRNA levels, measured by RNase protection assay (Fig. 2; Gore et al., 2000b). This finding is consistent with that from Naomi Rance's laboratory demonstrating that GnRH mRNA levels increase in postmenopausal compared to premenopausal women (Rance and Uswandi, 1996). Other studies, however, have shown age-related decreases in GnRH mRNA levels in intact (but not castrate) male F344 rats (Gruenewald and Matsumoto, 1991), and in 18 month compared to 2 month old female Sprague-Dawley rats (Li et al., 1997). Moreover, GnRH gene expression has been reported to decrease during aging in ovariectomized female rats, and we have made this observation in my laboratory as well (Rubin et al., 1997; Miller and Gore, 2001). Thus, the ovarian status of the animal is extremely important for the regulation of GnRH gene expression during aging. This may be much more complicated than just effects of ovarian steroid hormones, as the ovaries contain other non-steroid peptides and proteins that could influence the neuroendocrine system during aging.

The preovulatory GnRH/LH surge is attenuated in intact middle-aged compared to young rats on proestrus, and the steroid-induced GnRH/LH surge is also diminished with aging in ovariectomized female rats (Steger et al., 1980; Wise, 1984; Nelson et al., 1992; Rubin and King, 1994). My laboratory addressed the question of the preovulatory GnRH/LH surge in young and middle-aged female Sprague-Dawley rats. We had previously reported that GnRH mRNA levels increase significantly on the afternoon of proestrus in young female rats, at approximately 1500 h, shortly before the GnRH/LH surge which occurs at approximately 1800 h (Gore and Roberts, 1995). We examined this phenomenon in middle-aged rats, and found that the increase in GnRH mRNA levels in young rats is not seen in the middle-aged animals (Gore et al., 2000b). This result is consistent with the other changes in GnRH neurons

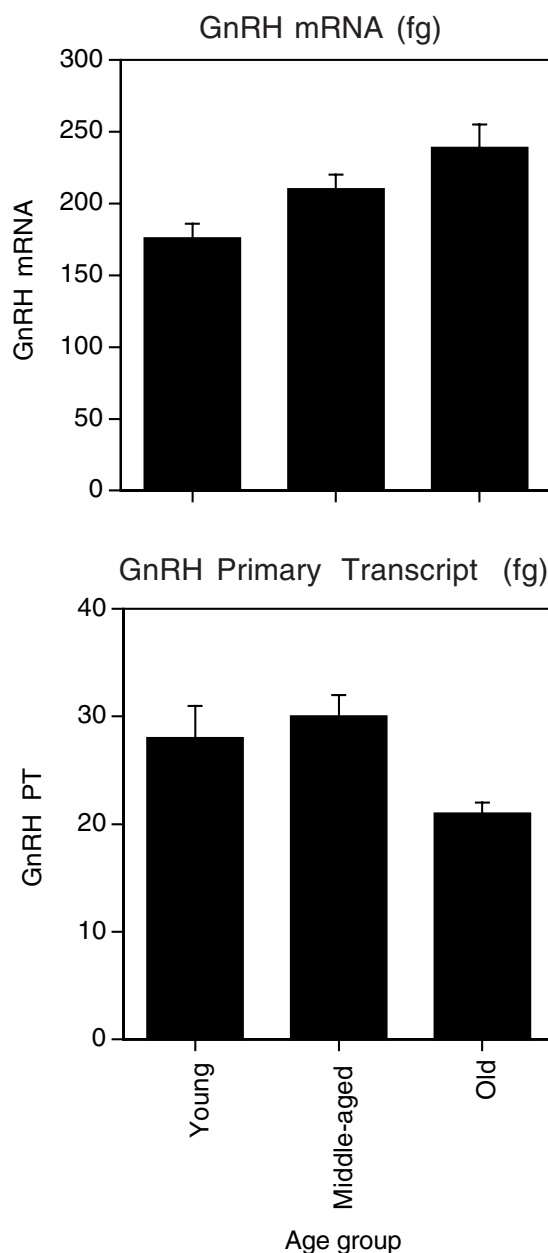


Fig. 2. Changes in GnRH mRNA (top) and primary transcript (bottom) during reproductive senescence in intact female rats. GnRH mRNA levels in the POA (expressed in fg) increase significantly during aging. GnRH primary transcript levels in the POA decrease during aging. Modified from Gore et al., 2000b.

discussed above, and may represent a substrate for the attenuation of the preovulatory GnRH/LH surge that occurs in rats, prior to reproductive failure.

NMDA receptor regulation of GnRH gene expression during aging

The regulation of the GnRH system by glutamate, acting through its NMDA receptor, changes across the reproductive life cycle. As discussed above, NMDA receptor activation stimulates GnRH release and elevates GnRH mRNA levels in young rats. A role for the NMDA receptor has also been suggested in reproductive senescence. The effects of NMDA receptor agonists on GnRH release in vivo and in vitro decrease during aging in rats (Arias et al., 1996; Zuo et al., 1996; Bonavera et al., 1998). My laboratory compared effects of NMDA receptor activation on GnRH mRNA levels between young and middle-aged rats (Gore et al., 2000a). We reported that treatment with an NMDA receptor agonist increases GnRH mRNA levels in young rats, similar to earlier studies (Petersen et al., 1991; Liaw and Barracough, 1993; Gore and Roberts, 1994). In contrast, in middle-aged female rats, the NMDA receptor agonist actually *decreases* GnRH mRNA levels (Gore et al., 2000a). Thus, the GnRH neurosecretory system of rats varies in its responsiveness to NMDA receptor activation depending upon the age and reproductive status of the animal.

Insulin-like growth factor-I (IGF-I) regulation of GnRH neurons

IGF-I is another factor that regulates the GnRH neurosecretory system. This neurotrophic factor is synthesized in peripheral organs such as liver, and is also produced in the brain, including the hypothalamus and median eminence (Garcia-Segura et al., 1991; Miller and Gore, 2001). The IGF-I receptor is also expressed in the brain, again including hypothalamus (Werther et al., 1989; Marks et al., 1991; Pons et al., 1991), suggesting that central IGF-I may act as an autocrine or paracrine factor regulating hypothalamic functions. With respect to the hypothalamic–pituitary–gonadal axis, IGF-I can stimulate LH release and advance the timing of puberty; also, circulating IGF-I levels increase during puberty (Hiney and Dees, 1991; Hiney et al., 1996; Wilson, 1998; Suter et al., 2000). Thus, IGF-I is thought to be a metabolic signal that is associated with the timing of reproductive maturation. Moreover, because IGF-I is

a target of the growth hormone axis, it may serve to coordinate the timing of the pubertal growth spurt and the onset of reproductive function.

We measured IGF-I mRNA levels in POA and medial basal hypothalamus (MBH) of rats undergoing reproductive maturation and reproductive aging. Using the RNase protection assay, we found that IGF-I mRNA levels in these two regions increase during pubertal development, and decrease during reproductive aging (Miller and Gore, 2001). In that same study, we examined effects of administration of IGF-I on GnRH mRNA levels, but did not see any effects in vivo (Miller and Gore, 2001). However, another study using hypothalamic explants from mice undergoing pubertal development showed that GnRH gene expression is stimulated by IGF-I under these in vitro conditions (Daftary et al., 2001). Differences between these results may be attributable to the experimental models. In the perfused hypothalami, IGF-I is applied to the bath, and can probably penetrate the tissue efficiently to exert its effects on the GnRH neurons (Daftary et al., 2001). In the in vivo studies, IGF-I is injected into the POA in vivo, but because this is a large region and GnRH neurons are dispersed throughout the POA, OVLT, septum and anterior hypothalamus, it is quite likely that IGF-I is not reaching all the target GnRH cells (Miller and Gore, 2001). Therefore, future studies are necessary to confirm whether IGF-I can stimulate GnRH gene expression in vivo as well as in vitro.

Post-transcriptional regulation of GnRH mRNA levels

Studies on the regulation of GnRH gene expression have led us to hypothesize that GnRH mRNA levels are predominantly regulated by a post-transcriptional mechanism in vivo. First, levels of GnRH nuclear transcripts are relatively high compared to other neuroendocrine genes, indicating that there is a large, steady-state pool of GnRH primary transcript that can result in the rapid accumulation of GnRH mRNA in the cytoplasm (reviewed in Gore and Roberts, 1997). Second, GnRH mRNA levels change extremely quickly in response to experimental manipulations, such as activation by NMDA receptor agonists, demonstrating the capacity for such a rapid accumulation (Petersen et al., 1991; Liaw and Bar-

raclough, 1993; Gore and Roberts, 1994). Third, changes in GnRH mRNA often occur in the absence of any concomitant changes in GnRH gene transcription (Gore and Roberts, 1994, 1995, 1997; Petersen et al., 1996). Fourth, GnRH mRNA turnover can occur very rapidly (Maurer and Wray, 1997). Based on these observations, it is proposed that the major mechanism for GnRH mRNA regulation occurs via a post-transcriptional mechanism, probably via an enhancement of GnRH mRNA stability. This may involve a decrease in the degradation of the cytoplasmic GnRH mRNA pool, through changes in the poly (A) tail length of the GnRH mRNA. The poly (A) tail size is thought to be an important indicator of mRNA stability, and is clearly implicated in the regulation of GnRH mRNA levels in the GT1-7 cell line (Gore and Roberts, 1997; Gore et al., 1997). Preliminary results suggest that NMDA receptor activation increases, and blockade decreases, GnRH mRNA poly (A) tail size in rats, indicating a similar phenomenon occurs in vivo as in the GT1 cells (Sun et al., 1996).

Neuroanatomical studies on GnRH neurons

Pharmacological studies demonstrate that GnRH-1 neurons receive inputs from numerous neurotransmitters, neurotrophic factors, and sex steroid hormones. GnRH neurons express receptors for many of these substances, making them likely targets for direct effects, and in addition, they undoubtedly receive indirect inputs that are mediated by interneurons. Nevertheless, several puzzles remain about the neuroanatomical regulation of the GnRH-1 neurosecretory system. In particular, the number of synaptic inputs to GnRH perikarya in the preoptic area (POA) and rostral hypothalamus is extremely small. Relatively few synapses are found on GnRH perikarya, with only 1–3 synapses per GnRH cell detected in rostral hypothalamus and POA of the rat (Witkin, 1989; Chen et al., 1990). Only approximately 1% of the total GnRH neuronal membrane is postsynaptic, which is significantly lower than that of other non-GnRH neurons in the same brain regions (Witkin, 1989). The number of axodendritic synapses onto GnRH neurons is also low, with ~4 and 3 synapses per GnRH dendrite found in females and males, respectively (Chen et al., 1990). A study on the rhesus monkey confirmed the paucity of synapses on GnRH

perikarya (from 2 to 12 per cell; Witkin et al., 1995). Thus the question remains as to how those receptors found on GnRH neurons may be exerting their effects, and if they may be non-synaptic in nature.

Hypothalamic NMDA receptors and their co-localization on GnRH neurons

NMDA receptors (NMDARs) are pentameric in structure and contain the NMDA-R1 (NR1) subunit, which is obligatory for a functional receptor (Ishii et al., 1993). A homomeric NR1 subunit is not fully functional, so that the NR1 must combine with at least one member of the NR2 family (consisting of NR2a–d; Monyer et al., 1992). The stoichiometry of the NMDAR is important in determining its functional properties in terms of glutamate binding, ion flux and signal transduction (Buller et al., 1994; Tang et al., 1999). The functions of the NMDAR are further complicated by the observation that the NR1 subunit has alternative splice variants that can confer further different structural and functional properties (Kusiak and Norton, 1993; Laurie et al., 1995). In POA, at least 7 NR1 splice variants have been detected (Laurie et al., 1995).

My laboratory and others have been characterizing the expression of NMDAR and non-NMDAR subunits on hypothalamic GnRH neurons, and their regulation. In developing rats, NR1, NR2a and NR2b mRNA are detectable in the POA, and their levels increase during the first two weeks of postnatal life (Adams et al., 1999). Jennes' laboratory reported the presence of NR1, NR2a and NR2b mRNA labelled cells in septum, diagonal band and POA (Eyigor and Jennes, 1996, 1997). During reproductive aging, NR1 mRNA levels do not change much, while NR2a and NR2b mRNA levels decrease during the transition to acyclicity (Gore et al., 2000a). Therefore, it is likely that the stoichiometry of the NMDAR may change concomitantly with reproductive senescence.

The issue of whether GnRH neurons express NMDARs is extremely controversial. Initial studies looking for co-localization of GnRH with NMDARs found only very low levels (Abbud and Smith, 1995; Eyigor and Jennes, 1996, 1997). In 1996, using an improved antibody to NR1, we reported that ~20% of GnRH neurons of adult female rats express NR1 (Gore et al., 1996). More recent studies using

electrophysiological recordings of identified GnRH neurons demonstrate that 20% of GnRH neurons respond to NMDAR activation with depolarization (Spergel et al., 1999). That same laboratory reported that all GnRH cells respond to glutamate, indicating that all GnRH neurons express either NMDA or non-NMDA receptors (Spergel et al., 1999). More recently, using techniques with greater sensitivity and improved antibodies, my laboratory reported that high percentages (50–80%) of GnRH neurons express NR1, NR2a and NR2b in young adult and middle-aged rats (Miller and Gore, 2002).

GnRH neuroterminals in the median eminence also coexpress NMDARs. Electron microscopy demonstrates that GnRH terminals co-localize with NR1 and non-NMDAR (KA2) receptors at the level of the median eminence (Kawakami et al., 1998a,b). Preliminary data from my laboratory, using postembbed immunogold double-label electron microscopy, indicate that the GnRH and NMDA receptor molecules are co-localized in the same neuroterminals, and moreover, that they are actually co-expressed within the same dense-core vesicles (Fig. 3). This is a novel localization of the NMDAR that is under investigation in ongoing physiological and electron microscopic studies in my laboratory.

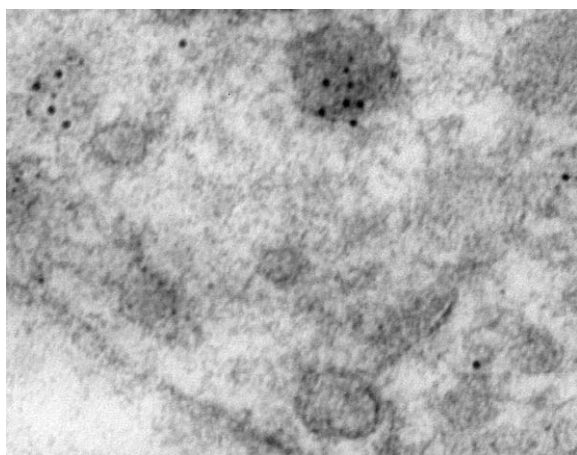


Fig. 3. Expression of the NMDA receptor in large dense-core vesicles in a GnRH neuroterminal in the median eminence. Double-label immunocytochemistry was performed using postembbed immunoelectron microscopic techniques. NMDA-R1 is identified with the larger (15 nm) gold particles, and GnRH with the smaller (5 nm) gold particles.

Insulin-like growth factor-I (IGF-I) co-localization in GnRH neurons

IGF-I is synthesized in the central nervous system in glia and neurons (Rotwein et al., 1988; Baxter et al., 1989; Garcia-Segura et al., 1991; Miller and Gore, 2001). The protein and mRNA of IGF-I and the IGF-I receptor are easily detectable in hypothalamus and median eminence (Werther et al., 1989; Garcia-Segura et al., 1991; Marks et al., 1991), demonstrating synthesis in regions proximal to GnRH perikarya and neuroterminals. A physiological role for IGF-I in reproductive function has also been shown. IGF-I enhances GnRH release from rat median eminence explants in vitro (Hiney et al., 1991), and LH levels in female rhesus (Wilson, 1998). Stimulatory effects of IGF-I are also seen on GnRH-induced LH secretion (Kanematsu et al., 1991).

My laboratory has been investigating the anatomical relationship between IGF-I in the brain and GnRH neurons. Because of its stimulatory effect on GnRH release, we predicted that cells expressing IGF-I would be found in the vicinity of GnRH neurons. To our surprise, we observed that a high percentage (~75%) of GnRH neurons in adult mouse and rat brain co-express IGF-I (Daftary et al., 2001; Miller and Gore, 2001; Fig. 4). The co-expression of IGF-I in GnRH neurons is subject to developmental regulation. Thus, the percentages of GnRH neurons expressing IGF-I increase from approximately 20 to 50 to 75% in neonatal, pubertal, and adult mice, respectively (Daftary et al., 2001). We speculate that GnRH neurons may synthesize IGF-I, and that this increases during maturation. This IGF-I in GnRH cells may serve an autocrine role regulating GnRH function, or may act as a paracrine factor in the hypothalamus regulating other non-GnRH cells. Two possible candidates for this action are the somatostatin or the growth hormone-releasing hormone neurons, both of which are sensitive to IGF-I regulation.

Conclusions

The regulation of GnRH neurons involves alterations in gene expression and neuroanatomical inputs. GnRH gene expression changes in response to stimuli such as steroid hormone feedback, varies across the estrous cycle, and changes across the

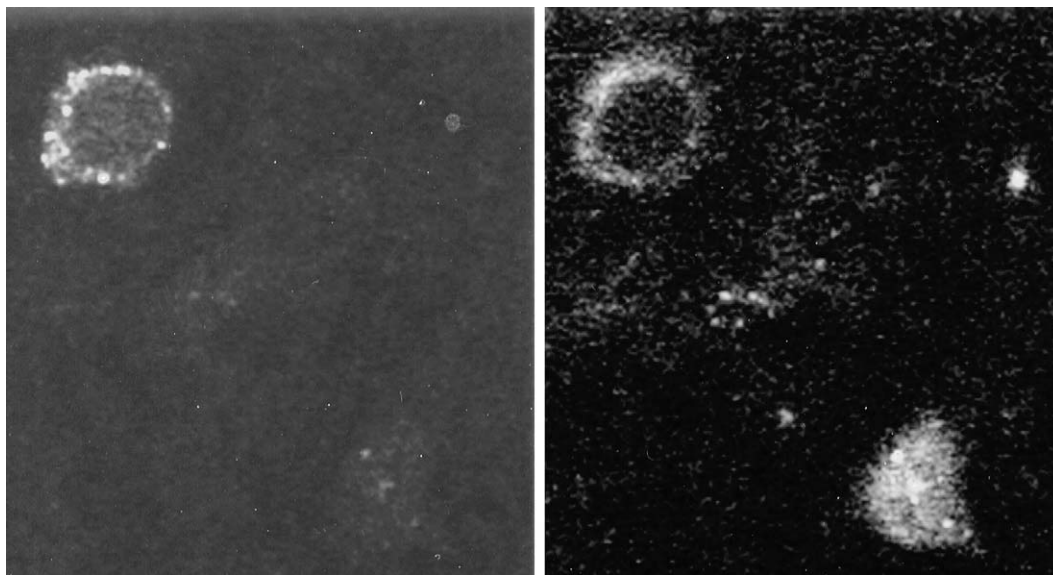


Fig. 4. Co-expression of IGF-I in a GnRH neuron of an adult female rat. A GnRH positive cell is shown in the left panel (upper left corner); the same cell, double-labeled with IGF-I, is shown in the right panel. Modified from Miller and Gore, 2001.

reproductive life cycle, particularly during pubertal maturation and reproductive aging. The most dramatic alterations in GnRH mRNA levels are seen during reproductive development in association with the pubertal process. Studies on the mechanisms for the regulation of GnRH mRNA levels suggest that a post-transcriptional process, involving an alteration in GnRH mRNA stability, possibly mediated by the GnRH poly (A) tail length, may be the primary one responsible for the control of GnRH mRNA levels. Inputs to GnRH neurons arising from neurotransmitters such as glutamate, and neurotrophic factors such as IGF-I, can affect the physiology and gene expression of the GnRH neurosecretory system. We observed that the expression of NMDA receptors on GnRH neurons is developmentally regulated, and that the responsiveness of GnRH cells to NMDA receptor activation is highest in pubertal animals and young adults, but decreases during reproductive senescence. For IGF-I, we found that GnRH cells express IGF-I, and again, this is developmentally regulated, with increases in co-localization occurring during reproductive maturation. Taken together, these studies demonstrate that changes that are intrinsic to GnRH neurons, as well as arising from extrinsic inputs to GnRH neurons, are integrated at

the level of the GnRH neuron to result in the appropriate reproductive physiology of the organism.

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GnRH pre-mRNA splicing: role of exonic splicing enhancer

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Introduction

Gonadotropin-releasing hormone (GnRH) plays a key role in the regulation of reproduction (Seeburg et al., 1987). GnRH is synthesized in the preoptic area (POA) of the vertebrate brain and released from nerve terminals in the median eminence into the hypothalamic–pituitary portal vessel in a pulsatile fashion. GnRH then acts on gonadotrope in the anterior pituitary to stimulate the biosynthesis and secretion of pituitary gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH).

The biosynthesis of GnRH can be regulated at multiple levels such as transcription rate, mRNA stability, translation rate of the mRNA into proGnRH peptide, and processing of the precursor to the mature GnRH decapeptide (Gore and Roberts, 1997; Gore et al., 1997). Recently, processing of the GnRH pre-mRNA to the mature mRNA has been reported as an important step for the regulation of GnRH biosynthesis. Various transcripts can be produced from a single GnRH gene by alternative RNA splicing (Seeburg and Adelman, 1984; Radovick et al., 1990; Zhen et al., 1997). Alternative splicing is often involved in functional diversification of proteins and quantitative control of gene expression (Lopez, 1998;

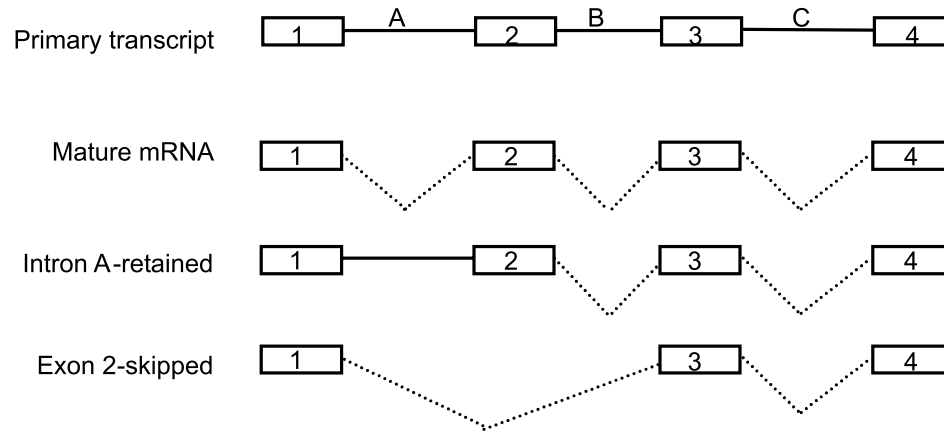
Lou and Gagel, 2001). Multiple transcripts generated by alternative splicing can serve as templates for producing various proteins that show different enzymatic function, subcellular localization, binding affinity to ligands, and electrophysiological properties (Hollmann et al., 1993; Kilpatrick et al., 1999). Generation of different 5' or 3' untranslated regions by alternative splicing can affect RNA stability and translational efficacy (Wang et al., 1999). Recently, we and others found that, in contrast to the mature mRNA, GnRH splice variant transcripts exhibit a severe defect in producing the GnRH peptide (Zhen et al., 1997; Seong et al., 2001). Thus, elucidation of the detailed mechanism for alternative GnRH pre-mRNA splicing is quite important for understanding the regulation of GnRH biosynthesis. In this article, we will discuss the recent progress in research regarding the enhancer-dependent GnRH pre-mRNA splicing mechanism, the possible *trans*-acting factors that may act on the splicing enhancer element, and the physiological relevance of enhanced and/or attenuated GnRH pre-mRNA splicing.

Alternative splicing of the GnRH pre-mRNA

The GnRH gene consists of 4 short exons (denoted 1, 2, 3, and 4) and 3 long introns (A, B, and C) (Mason et al., 1986; Bond et al., 1989). In the hypothalamus and GnRH-producing cell lines, all three introns are excised from the primary transcript (~4,300 bases), producing a mature mRNA of about 560 bases (Adelman et al., 1986). Despite low

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A



B

mouse intron A GTTTCTGTGAAGCCAGTGGTCCAGGAGCTGGCCTCTTTGCTAAGCA TTCTTC**ACTCTGTGTC**TTGAT**GTCCCTTAG**
rat intron A GTTTCTGTGGAGTCAGTGGTCCGGGTGCTGGCTTCTTTCCAAAGTA TTCTTCCCTCTGTGTCTTGAT**GTCCCTTAG**
human intron A TTTTCACTAAAGAGGTCTTTTAGTTTATACTCAACCTTGCTGGATCT **AA**TTGATGTGCATTCA**TGTGCCTTAG**

mouse intron B CACAGACAAGTCTTGGTG**CAAAT**GGAAACTGTTTGTGTTTACCTTCATGTTTGAGGG TCTCATTCTTT**ATCTCCAG**

mouse intron C TTTTAGAAAAACGTCAGAAACAATGAAATTCTTCAGCTAGTCCGT**CAAC**TTTCATAAGCA TGTTTT**ATA**ATTTTCAG

Fig. 1. (A) Alternative GnRH pre-mRNA splicing. From a single GnRH gene, three GnRH RNA species (mature mRNA, intron A-retained, and exon 2-skipped RNA species) could be produced by alternative RNA splicing. Boxes and lines indicate GnRH exons and introns, respectively. Dashed lines indicate the excision of introns and/or exons during alternative splicing. (B) The 3' splice sites of introns A, B, and C of the GnRH gene. Underlines indicate the pyrimidine tract. Purine bases in the pyrimidine tract were indicated in bold. Boxes represent the putative branch point site (YNRAY). Note that the putative branch point sites are within the pyrimidine tract of intron A of the mouse, rat and human GnRH gene.

level expression, GnRH transcripts are also found in the peripheral tissues including reproductive and immune-related tissues, and in some discrete brain regions such as olfactory bulb and piriform cortex (Radovick et al., 1990; Azad et al., 1991; Pagesy et al., 1992; Choi et al., 1994). Interestingly, RNA processing in extra-hypothalamic tissues appears to differ from that observed in the hypothalamus. It is noteworthy that the GnRH cDNA from the human placenta contains the first intron (intron A) (Seeburg and Adelman, 1984). Subsequent studies demonstrated that non-hypothalamic tissues, such as the placenta and reproductive organs prevalently express the intron A-containing RNA species than the mature mRNA (Radovick et al., 1990; Dong et al., 1996; Seong et al., 1999). Moreover, a GnRH transcript lacking exon 2 has been found in Gn11 cells and immune cells (Wilson et al., 1995; Zhen et al., 1997). Thus far, all of the three RNA species (intron A-retained, exon 2-skipped, and mature mRNA) have been shown to be produced from a single GnRH gene (Fig. 1A). However, the mechanism for producing three transcripts has been poorly understood. In order to better understand the molecular events, Roberts and his colleagues initiated an investigation of the GnRH pre-mRNA splicing mechanism using a sensitive RNase protection assay and RT-PCR (Jakubowski and Roberts, 1994). They found relatively high level of the intron A-containing RNA species, but low level of the intron B- and intron C-containing RNA species in the nuclear fraction, indicating the notion that introns B and C are rapidly removed from the primary transcript, while intron A is slowly excised. Consistent with this result, we recently demonstrated that, using an *in vitro* RNA splicing system, intron A could not be removed, while introns B and C were easily excised (Fig. 2) (Seong et al., 1999). These findings indicate that intron A excision is a rate limiting step for GnRH pre-mRNA splicing.

The *cis*-elements essential for intron excision are located in the exon-intron joint. At the downstream side of an exon, the 5' splice site in higher eukaryotes conforms to the consensus sequence, AG/GURAGU (the splice site is denoted by a slash and invariant nucleotides are underlined; R = purine, Y = pyrimidine, N = any nucleotide). At the upstream side of the exon, the 3' splice site conforms to consensus,

Y_(n)AG/G in most vertebrate introns (Shapiro and Senapathy, 1987). The branch point site (BPS) is usually located at a distance of 18 to 40 nucleotides upstream the 3' splice site with very loose consensus, YNYRAY (the site of branch formation is underlined) (Berglund et al., 1997). The splice sites are catalyzed by numerous proteins in a macromolecular complex, the spliceosome complex that consists of small nuclear ribonucleoproteins (snRNPs), heteronuclear RNPs (hnRNPs), and a large family of serine/arginine-rich proteins (SR proteins) (Zuo and Maniatis, 1996). The spliceosome complex would barely interact with the suboptimal splice site, while strongly interacting with well-conserved splice sites. Thus, it is proposed that the attenuation of intron A excision from the GnRH pre-mRNA is most likely due to suboptimal splice sites of intron A. Sequence analysis of the 3' splice sites of introns A, B, and C of the GnRH gene revealed that the 3' splice site of intron A contains many purines in the pyrimidine tract, whereas 3' splice sites of introns B and C are highly conserved (Seong et al., 1999) as other normal 3' splice sites (Shapiro and Senapathy, 1987). Moreover, a putative BPS is located within the pyrimidine tract but not in the upstream side of the pyrimidine tract (Fig. 1B). The 3' spliceosome complex formation begins with U2 auxiliary factor (U2AF) recognizing the pyrimidine bases in the pyrimidine tract, thereby U2 snRNP binds to the 3' splice site. However, many purines in the pyrimidine tract could interfere with binding to U2AF (Lavigne et al., 1993; Tian and Maniatis, 1994). The BPS is usually located upstream the pyrimidine tract and recognized by branchpoint bridging protein (BBP) (Berglund et al., 1997). BPS within the pyrimidine tract may cause a steric hindrance of BBP and U2AF binding to the pyrimidine tract. Thus, the presence of many purine bases in the pyrimidine tract and the abnormal location of BPS appears to be responsible for the attenuation of intron A excision. This possibility is strongly supported by the observations that either mutations of purines to pyrimidines or translocation of the putative BPS to upstream of the pyrimidine tract significantly increased the splicing rate (Seong et al., 1999). The increased splicing activities caused by the mutation of the suboptimal site were also observed in growth hormone (GH) and doublesex (*dsx*) pre-mRNA splicing (Tian and Maniatis, 1994;

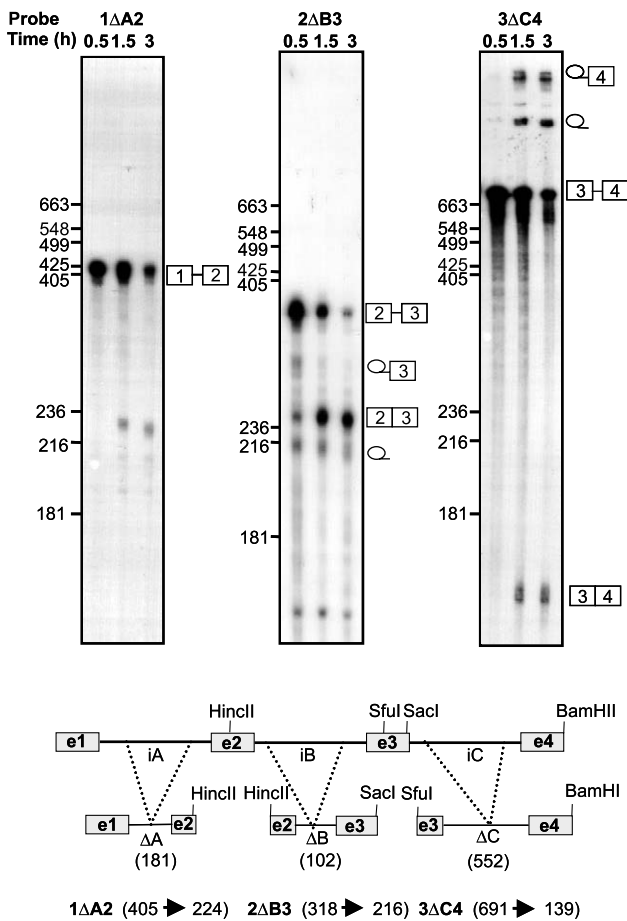


Fig. 2. Excision rate of rat GnRH introns in vitro. The ^{32}P -labeled RNA substrates consisting of each GnRH intron and its neighboring exons were synthesized by in vitro transcription. The RNAs purified from the 6% polyacrylamide gels containing 8 M urea were incubated with HeLa NE. The products were electrophoresed on the 6% polyacrylamide gels containing 8 M urea and then subjected to autoradiography. RNA substrates are shown at the top of the gels and the time of incubation is specified at the top of each lane. Illustrated RNA structures on the right sides of gels were identified by RNA size markers and by running on higher percentage gels. Spliced RNA (exon-exon) was confirmed by RT-PCR using gel-purified RNAs. Other bands in this and following figures are cryptic splicing intermediate(s) and denoted with asterisks. The schematic diagram on the bottom panel shows the restriction map of the GnRH gene and RNA substrates. The sizes of substrates containing each intron and their spliced products are shown in parentheses.

Dirksen et al., 1995). Although the 5' splice site of intron A (GUAAAA) is not well conserved as other normal 5' splice sites (GURAGU), it seems not to affect intron A excision since the mutation of the 5' splice site of intron A toward consensus did not significantly increase intron A excision (Seong et al., 1999). Exon 2 skipping from the GnRH pre-mRNA could be explained by the same context as intron A retention. Since the 3' spliceosome complex could not be formed on intron A, a 3' spliceosome complex that is formed on the 3' splice site of intron B can

directly interact with the 5' spliceosome complex on the 5' splice site of intron A, which allows exon 2 to be removed.

Exonic splicing enhancers (ESEs) in GnRH exons 3 and 4

The presence of a 3' suboptimal splice site in intron A raises a fundamental question of how GnRH-producing cells generate the mature mRNA. It is well known that the suboptimal splice site could be

actively recognized by the 3' spliceosome complex in the presence of additional positive exon element, such as purine-rich enhancer (Tian and Maniatis, 1992; Sun et al., 1993; Tanaka et al., 1994). Recently, list of enhancers has been extended to include the AC-rich sequences (Coulter et al., 1997) and certain intronic sequences (Modafferi and Black, 1997). In addition to their role in constitutive pre-mRNA splicing, SR proteins are known to be involved in enhancer-dependent splicing by binding to their specific enhancer element (Tian and Maniatis, 1994; Liu et al., 1998; Schaal and Maniatis, 1999). Thus, it is postulated that the presence of additional enhancer elements in the GnRH pre-mRNA and a specific splicing factor(s) acting on the enhancer elements is a prerequisite for achieving the mature GnRH mRNA in GnRH-producing cells.

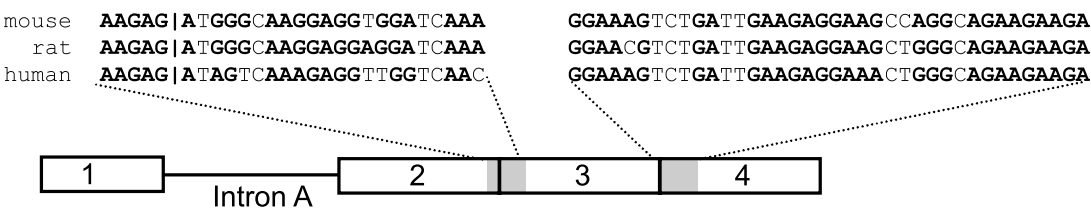
Sequence analysis of GnRH exons reveals that two purine-rich regions are located in exon 3 and exon 4 (Seong et al., 1999). The sequence of purine-rich regions in GnRH exons exhibits an extremely high degree of sequence similarity among mouse, rat, and human (Fig. 3A). Purine-rich regions in exons 3 and 4 exhibit alternative repeats of guanosine (G) and adenosine (A) bases, like GAR (R = purine) or AGR. Concerning rapid and efficient excisions of introns B and C, it is postulated that exons 3 and 4 can join to exon 2, producing the 1A234 RNA species. Then, the majority of the 1A234 RNA species undergo further processing of intron A excision with the help of exonic splicing enhancers (ESEs) located in exons 3 and 4. This possibility is strongly supported by the finding that, in an *in vitro* RNA splicing system, intron A is partially excised when exon 3 and exon 4 are linked up with exon 2, while intron A was not excised at all in the presence of exon 2 alone. The partial excision of intron A from the 1A234 construct is attributable to the distance of the purine-rich sequences in exons 3 and 4 from the 3' splice site of intron A. The purine-rich sequences show a strong enhancing activity when they are located close to the 3' splice site of intron A (Fig. 3B). Thus, the closer the purine-rich sequence to the 3' splice site of intron A, the better the splicing activity (Seong et al., 1999), which is a common feature of ESE found in other pre-mRNA splicing (Tanaka et al., 1994). Mutations in the purine-rich sequences in exons 3 and 4 decreased pre-mRNA splicing activity, while

mutation in the purine-rich sequence in exon 2 did not affect the splicing activity. These results suggest that the purine-rich sequences in exons 3 and 4 are functionally active ESEs, which are denoted ESE3 and ESE4, respectively (Seong et al., 1999). ESE4 consists of three purine repeats separated by two spacers and a putative hairpin constructing sequence. Analysis of twelve ESE4 mutants suggest that there are at least three parameters affecting intron A excision (Han et al., 2001). First, the amount of the purine residues in the purine-rich region is critical for the enhanced splicing activity. The purine-rich sequence in the middle of exon 2 contains 14 purines while ESE3 and ESE4 contains 22 and 30 purines, respectively. Enhancing activity of ESE4 was greater than ESE3, and the purine-rich sequence in exon 2 did not serve as an enhancer. The decrease in purine number in ESE4 significantly reduced enhancing activity. Thus, a certain number of purine bases in ESE is required for maintaining the enhancing activity. Second, pyrimidine residues between two long continuous purine repeats seem to be stimulatory. An increase in splicing activity was observed when a longer pyrimidine spacer region between two long purine repeats was created by mutations of three purines in the spacer to pyrimidines. This result indicates that an optimal alignment of the purine repeats and pyrimidine spacer can improve the binding affinity to a certain splicing factor(s). Third, pyrimidine residues within the continuous purine repeats are deleterious, especially when they are located very close to 3' splice site. Mutations of purines in polypurine repeats to pyrimidines greatly reduced splicing activity, indicating that a certain number of purines (at least 6–8 purines) should be continuously repeated without interruptions by pyrimidine for enhanced splicing activity.

Presence of GnRH neuron-specific splicing factors acting on ESE4

Alternative pre-mRNA splicing relies on a large family of SR proteins. Up-to-date, more than ten SR proteins, SRp20, ASF/SF, SC35, SRp30, SRp40, SRp55, SRp75, 9G8, Tra, and Tra2 have been identified (Manley and Tacke, 1996). SR proteins are characterized by a modular composition, comprised of one or more RNA recognition motifs (RRM) and

A



B

RNA constructs		Splicing activity	
		HeLa	HeLa+GT1
1A2		–	–
m1A2		+++	ND
1A23		+	+
1A234		+	+++
1A3		+	+
1A4		+++	++++
1A34		+++	++++

Fig. 3. Exonic splicing enhancers (ESEs) and their role in GnRH pre-mRNA splicing. (A) Schematic representation of the GnRH pre-mRNA and ESEs. Gray boxes indicate exonic splicing enhancers (ESEs) in GnRH exons 3 and 4, and their sequences are shown above the RNA structure. Purine bases are shown in bold. The vertical bars indicate the border of exons 2 and 3. The purine-rich sequences of the mouse, rat, and human GnRH gene are well conserved. (B) Splicing activities of the GnRH pre-mRNA in the presence of ESE. RNA constructs are shown in the left and their splicing activities in HeLa or HeLa + GT1 nuclear extracts are shown in the right. Splicing activity indicates % splicing; splice product/(spliced product + precursor RNA). Each + indicates the about 10% splicing activity. Splicing activity was observed in the presence of ESEs or when 3' spliced site was mutated to the consensus (m1A2 construct). It should be noted that GT1 nuclear extract further increased splicing activity only in the presence of ESE in exon 4.

SR domain in which arginine and serine residues are repeated. It has been proposed that SR proteins can bind to each other using their SR domains, allowing protein–protein interaction to form networks of SR proteins (Lynch and Maniatis, 1996). SR proteins are expressed in all tissues (Zahler et al., 1993) and can complement splicing-deficient nuclear extracts, indicating that they are essential splicing factors for general or constitutive splicing (Zahler et al., 1992).

However, they appear to be equally important in alternative splicing being able to modulate selection of alternative splice sites in a concentration-dependent manner (Mayeda and Krainer, 1992; Caceres et al., 1994; Hanamura et al., 1998). Although earlier studies suggest that SR proteins might be functionally redundant, recent studies indicate that each protein probably performs at least some non-redundant functions (Tacke and Manley, 1999). Evidence for diver-

gent RNA binding specificity among SR proteins has been achieved by extensive application of SELEX (systemic evolution of ligands by exponential enrichment), which allows the identification of high-affinity binding sites from pools of random RNA sequence (Liu et al., 1998; Schaal and Maniatis, 1999).

In a HeLa nuclear extract (NE), the 1A234 pre-mRNA exhibited a partial splicing activity since ESEs are located at a far distance from the 3' splice site of intron A. Interestingly, addition of GT1 (GnRH-producing cell) NE further increased excision rate of intron A from the 1A234 pre-mRNA in a dose-dependent manner, while addition of KK1 (non-GnRH-producing cell) NE rather decreased it (Seong et al., 2002). These results are consistent with the *in vivo* experiments in which the most efficient excision of intron A was found in GT1 cells and the POA, while intron A-containing transcript was abundant in extrahypothalamic regions of the brain and peripheral tissues (Seong et al., 1999). There appears to be a GnRH neuron-specific *trans*-acting factor(s) interacting with ESE4. It is of importance to note that most splicing enhancers are active when located within 100 bases from the 3' splice site. However, ESE4 is located at 238 bases downstream the 3' splice site of intron A when exons 3 and 4 are linked up with the exon 2. Indeed, full enhancing activity of ESE4 was observed when ESE4 is close to the 3' splice site of intron A. This finding raises a question of how such an efficient splicing activity of the 1A234 pre-mRNA is achieved in GnRH-producing cells. One good example accounting for the unusually increased splicing activity of the 1A234 pre-mRNA is the *Drosophila melanogaster* dsx pre-mRNA splicing mechanism. The dsx pre-mRNA has the dsx repeat element(dsxRE) that normally functions when located at 300 bases downstream of the 3' splice site in the presence of some specific splicing factors, Tra and Tra2 (Lynch and Maniatis, 1995). In the absence of Tra/Tra2, these elements are not active when located at a long distance from the 3' splice site. Tra/Tra2 may form a stable complex with dsxRE, recruiting a certain splicing factor to allow the enhancer to function at this distance (Lynch and Maniatis, 1995, 1996). Recently, two human homologs of Tra2, Tra2 α (Dauwalder et al., 1996) and Tra2 β (Beil et al., 1997) have been

identified. Interestingly, the purified Tra2 proteins can bind preferentially to RNA sequences containing GAA repeats (Tacke et al., 1998) that are also seen in ESE4 of the GnRH pre-mRNA. Northern blot analysis for mouse Tra2 α indicates that the POA and GT1 cells express Tra2 α mRNA, suggesting that Tra2 α may participate in enhanced splicing activity of the 1A234 pre-mRNA. Indeed, we observed that purified GST-tagged Tra2 α protein actively bound to the ESE4 sequence, while several other splicing factors did not (Seong et al., 2002). Moreover, Tra2 α did not bind to mutant ESEs in which purines were changed to pyrimidines. This result indicates that Tra2 α specifically binds to purine stretches in the ESE4 sequence, and then probably forms a stable complex with other SR protein(s) or GnRH neuron-specific splicing factor(s), allowing ESE4 to help intron A excision at a long distance. It is notable that enhanced splicing of the GnRH pre-mRNA was observed when a nuclear fraction of GnRH neurons that was precipitated at the saturation of 40–50% ammonium sulfate (ASP40–50) was added to splicing reaction (Seong et al., 2002). ASP40–50 fraction significantly increased the excision rate of intron A in the presence of HeLa NE or SR protein-rich fraction. However, ASP40–50 fraction alone could not remove intron A. Activity of ASP40–50 fraction was much greater in RNA constructs in which ESE4 is far from the 3' splice site of intron A than in RNA constructs in which ESE4 is close to the 3' splice site of intron A or RNA construct which is constitutively active in HeLa NE. This result suggests that a splicing factor(s) in ASP40–50 fraction is not necessary for constitutive RNA splicing but required for splicing of the pre-mRNA in which ESE is far from the 3' splice site. A splicing factor(s) in ASP40–50 fraction seems not to bind ESE4 directly as revealed by UV cross-linking and affinity chromatography studies. This result indicates the splicing factor(s) in ASP40–50 fraction is a cofactor protein(s) that is likely to interact with the SR protein, probably Tra2 α that is already bound to ESE4. We recently performed a yeast two hybrid assay using Tra2 α as a bait from the GT1-1 cDNA library. One of the SR proteins, SRp30c was isolated and confirmed to bind to Tra2 α . Therefore, such an interaction appears to be important for the GnRH neuron-specific enhancement of GnRH pre-mRNA splicing as schematically presented in Fig. 4.

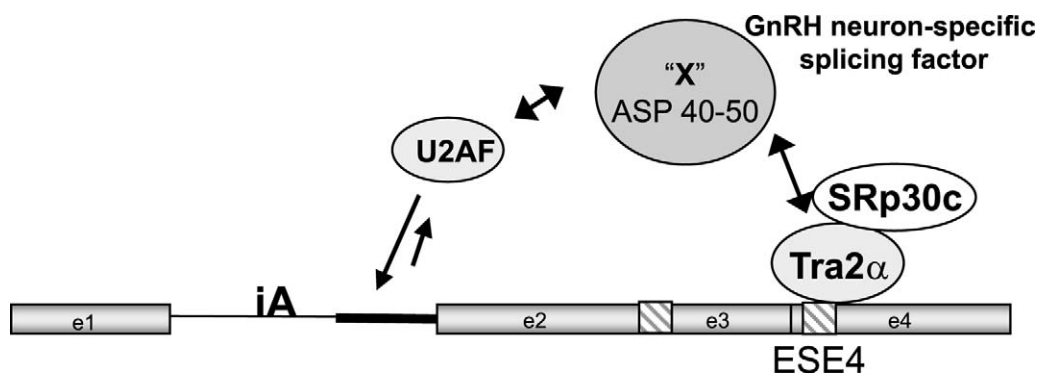


Fig. 4. A possible model for the enhanced splicing of the GnRH pre-mRNA. Efficient splicing of the GnRH pre-mRNA may be mediated by an interaction of ESE4 and ESE4-recognizing splicing factors in GnRH neurons. A 40 kDa protein, Tra2 α specifically binds to ESE4. A cofactor protein (X) in ASP40–50 fraction seems to interact with Tra2 α that is already bound to ESE4. Interaction of a cofactor protein(s) in ASP40–50 fraction with ESE4–Tra2 α complex may recruit U2AF to recognize suboptimal 3' splice site of intron A, leading to an enhancement of GnRH pre-mRNA splicing. In addition SRp30c interacts with Tra2 α that is already bound to ESE4. This interaction of SRp30c with ESE4–Tra2 α complex also seems to recruit U2AF to recognize suboptimal 3' splice site of intron A, leading to an enhancement of GnRH pre-mRNA splicing.

Developmental changes in GnRH pre-mRNA splicing

Recent studies have demonstrated that hormonal inputs alter the splicing pattern of a variety of genes such as potassium channel, activin receptor II, myosin II heavy chain-B, dopamine D2 receptor, and GnRH receptor (Kawamoto, 1996; Cowley et al., 1998; Guivarc'h et al., 1998; Shoji et al., 1998; Xie and McCobb, 1998). Alterations in GnRH primary transcript level during mouse embryonic and postnatal development were not accompanied by similar changes in GnRH mRNA level (Gore et al., 1999). Similarly, uncoupling of transcription and posttranscription process of GnRH transcripts by steroids or neural inputs was also observed (Gore and Roberts, 1995). A potential explanation for the uncoupling of transcription and posttranscription, it has been suggested that the changes in splicing rate of the GnRH pre-mRNA may be, at least in part, involved in this process. Recently, we demonstrated that the excision rate of intron A in the POA was increased during postnatal development of normal mice, which was accompanied by an increase in mature GnRH mRNA levels (Seong et al., 2001). The intron A excision rate in the POA was significantly increased in 3-week-old mice and further increased until adulthood. In contrast, in the cortex (CTX), intron A excision rate was extremely low, further decreased

in 3-week-old mice, and remained at very low levels until adulthood. Excision rate of introns B and C in the POA was not significantly changed during development. GnRH mRNA levels in the POA gradually increased during development, similarly to the intron A excision rate. The developmental changes in intron A excision rate indicates developmental maturation of the splicing machinery to increase splicing rate of the GnRH pre-mRNA in GnRH neurons. Interestingly, mRNA levels for Tra2 can be altered by neural activation or hormonal input (Daoud et al., 1999). This finding suggests that changes in splicing regulator protein levels may affect the splicing patterns of the downstream pre-mRNAs (Stamm et al., 1999), raising the possibility that a variety of signaling may affect the expression of splicing factors in GnRH neurons, thereby allowing splicing pattern of the GnRH pre-mRNA to be altered.

Biological relevance of efficient intron A excision: analysis of hpg mouse

Splice variant transcripts produced by alternative splicing often have a distinct role compared with wild type transcripts. An alternative splice variant of growth hormone-releasing hormone (GHRH) transcripts generates a new C-terminal peptide (Perez-Riba et al., 1997). Fourth intron retention of growth hormone (GH) transcripts produces an isoform of

GH (Yamamoto et al., 1998). Alternative transcripts of nitric oxide synthase (NOS) having different 5' untranslated regions (5' UTR) exhibit differential translation efficiency (Wang et al., 1999). So, what is the physiological relevance of alternative splicing of the GnRH pre-mRNA? Hypogonadal (hpg) mice may provide a clue for such a question. Hpg mice are infertile due to lack of the GnRH biosynthesis in the hypothalamus (Mason et al., 1986; Seeburg et al., 1987). Hpg mice have a natural genetic defect of the GnRH gene of which exons 3 and 4 are truncated (Mason et al., 1986). Since no truncation or mutations in the GnRH promoter sequence has been observed in hpg mice and GnRH exon 2 that encodes a signal peptide, GnRH decapeptide, and a part of GnRH-associated peptide (GAP) is intact in hpg mice, despite the lack of exons 3 and 4, it could be postulated that hpg mice could produce the GnRH peptide. However, little GnRH is detectable in hpg mice (Mason et al., 1986). Thus, the reason for the low production of GnRH in the hpg mice is poorly understood. Recently, we found that intron A excision rate in the POA of hpg mice was severely lower than that of normal mice (Seong et al., 2001). Such an attenuation of intron A excision in hpg mice is most likely due to the lack of GnRH exons 3 and 4 in which ESEs are located. Our in vitro splicing assay using either HeLa or GT1 NE demonstrated that excision of intron A took place in the presence of exons 3 and 4, while exon 2 alone marginally affected the intron A excision (Seong et al., 1999). Thus, it may be proposed that low excision rate of intron A results in a larger amount of the intron A-retained transcript than the mature mRNA, and that the intron A-retained transcript has a severe defect in translation capacity, eventually leading to little biosynthesis of the GnRH peptide. In fact, sequence analysis reveals that intron A provides many AUGs and at least five conserved Kozak sequences (Kozak, 1987), suggesting the existence of multiple short open reading frames. The presence of multiple AUGs or Kozak sequences in intron A may, in part, interfere with the translation efficiency. In support of this idea, an insertion of the intron A sequence into the upstream region of the luciferase gene markedly decreased translation rate without affecting transcription rate (Seong et al., 2001). In addition to the intron A-retained RNA species, an alternative splicing variant

form of the GnRH transcript lacking exon 2 was found in the mouse olfactory, hypothalamus and immortalized GnRH cell lines (Zhen et al., 1997). Since exon 2 encodes the GnRH peptide, this RNA species also cannot produce the GnRH peptide. Thus, either intron A-retained or exon 2-skipped RNA species has a defect in producing the GnRH peptide.

In conclusion, in addition to transcription, translation, and posttranslational regulation, the efficient and accurate processing of the GnRH pre-mRNA is critical for tissue-specific and/or developmental stage-specific GnRH expression, thereby maintaining normal function of GnRH neurons. Identification of GnRH neuron-specific splicing factors that may interact with ESE provides important insights into the GnRH gene regulation mechanism.

Acknowledgements

The present study was supported by the Ministry of Science and Technology through Korea Brain Science Program and the National Research Laboratory (2000-N-NL-01-C-149). J. Han, and G.H. Son are recipient for the post-doctoral and pre-doctoral fellowship from Brain Korea 21 of the Ministry of Education of Korea, respectively.

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Pro-GnRH processing

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Introduction

The anterior pituitary has long been known to regulate stress, reproduction, lactation, growth, and metabolism, while the posterior pituitary controls osmolarity and fluid volume, milk ejection from the breast, and uterine contractions in labor (Reeves et al., 1998; Thorner et al., 1998). Due to its multiplicity of functions, the pituitary was thought to be the 'master gland'. In the early part of the twentieth century, Erdheim and Stumme (1909) used pathological analyses to show that pituitary insufficiency in humans could be produced by hypothalamic damage. Later, Aschner (1912) reported that hypothalamic lesions that spared the pituitary could disrupt reproductive functions in dogs. Despite these early studies, it was not until the classic experiments of Harris and Jacobsohn (1952) that an endocrine role for hypothalamic control of anterior pituitary function was shown. In this case, pituitary stalk section in the rat produced a loss of reproductive function and the condition was reversed when the hypophyseal blood vessels regenerated. Transplantation experiments further emphasized the importance of the hypothalamus in the control of reproduction

(Nikitovitch-Winer and Everett, 1958). Removal of the pituitary to the renal capsule or temporal lobe was unable to correct the deficit. However, when the pituitary was placed beneath the median eminence of the hypothalamus and revascularization occurred, reproductive function was restored. Additionally, transplantation of pituitary fragments into the mediobasal hypothalamus further demonstrated a trophic role for this region of the brain (Halasz et al., 1962; Knigge, 1962).

Since an intact hypophyseal-portal circulation was necessary to re-establish anterior pituitary function, many investigators began to try to identify these hypothalamic hormones or releasing factors. Using extracts from hypothalamus, McCann (McCann et al., 1960) and Harris (Campbell et al., 1964) identified a biologically active material that was able to stimulate the release of gonadotropin hormones from the pituitary. In 1971, Schally and coworkers (Matsuo et al., 1971; Schally et al., 1973) isolated and sequenced a decapeptide from thousands of porcine hypothalami and showed that this and the synthetic peptide could release luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary. These findings were replicated using extracts from ovine hypothalamus by Guillemín and collaborators (Burgus et al., 1972). The peptide with the LH- and FSH-releasing activities was termed gonadotropin-releasing hormone (GnRH) or luteinizing hormone-releasing hormone. This hormone contains 10 amino acids where the N-terminal residue consists of a pyroglutamate and the C-terminal has a glycine-amide (see Table 1). These two modifications serve to retard degradation of the peptide in

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TABLE 1

Molecular forms of GnRH

	Amino acid position ^{a,b}									
	1	2	3	4	5	6	7	8	9	10
mGnRH ^c	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	Gly-NH ₂
cGnRH-I	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Gln	Pro	Gly-NH ₂
sbGnRH	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Ser	Pro	Gly-NH ₂
fGnRH	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Trp	Pro	Gly-NH ₂
pjGnRH	pGlu	His	Trp	Ser	Phe	Gly	Leu	Ser	Pro	Gly-NH ₂
hrGnRH	pGlu	His	Trp	Ser	His	Gly	Leu	Ser	Pro	Gly-NH ₂
cfGnRH	pGlu	His	Trp	Ser	His	Gly	Leu	Asn	Pro	Gly-NH ₂
sGnRH	pGlu	His	Trp	Ser	Tyr	Gly	Trp	Leu	Pro	Gly-NH ₂
gpGnRH	pGlu	Tyr	Trp	Ser	Tyr	Gly	Val	Arg	Pro	Gly-NH ₂
cGnRH-II	pGlu	His	Trp	Ser	His	Gly	Trp	Tyr	Pro	Gly-NH ₂
dfGnRH	pGlu	His	Trp	Ser	His	Gly	Trp	Leu	Pro	Gly-NH ₂
lGnRH-III	pGlu	His	Trp	Ser	His	Asp	Trp	Lys	Pro	Gly-NH ₂
tGnRH-I	pGlu	His	Trp	Ser	Asp	Tyr	Phe	Lys	Pro	Gly-NH ₂
tGnRH-II	pGlu	His	Trp	Ser	Leu	Cys	His	Ala	Pro	Gly-NH ₂
lGnRH-I	pGlu	His	Tyr	Ser	Leu	Glu	Trp	Lys	Pro	Gly-NH ₂

^a The amino acids are designated by three-letter abbreviations; pGlu refers to pyroglutamate, Gly-NH₂ is glycine amide.

^b Amino acid differences from the mammalian GnRH are highlighted.

^c *Mammalian (m)*: Matsuo et al., 1971; Burgus et al., 1972; *chicken (c) I*: King and Millar, 1982; *seabream (sb)*: Powell et al., 1994; *frog (f)*: Yoo et al., 2000; *pejerrey (pj)*: Montaner et al., 2001; *herring (hr)*: Carolsfeld et al., 2000; *catfish (cf)*: Ngamvongchon et al., 1992; *salmon (s)*: Sherwood et al., 1983; *guinea pig (gp)*: Jimenez-Liñan et al., 1997; *chicken (c) II*: Miyamoto et al., 1984; *dogfish (df)*: Lovejoy et al., 1992; *lamprey (l) III*: Sower et al., 1993; *tunicate (t) I and II*: Powell et al., 1996; *lamprey (l) I*: Sherwood et al., 1986.

blood (Chertow, 1981).

Eleven years following the identification of the sequence for mammalian GnRH, a second form was isolated from chickens (King and Millar, 1982) and this was soon followed by the purification of another form from the same species (Miyamoto et al., 1984). Presently, fifteen different molecular forms of the decapeptide have been isolated and sequenced from a variety of different species of animals (Matsuo et al., 1971; Burgus et al., 1972; King and Millar, 1982; Sherwood et al., 1983, 1986; Miyamoto et al., 1984; Lovejoy et al., 1992; Ngamvongchon et al., 1992; Sower et al., 1993; Powell et al., 1994, 1996; Jimenez-Liñan et al., 1997; Carolsfeld et al., 2000; Yoo et al., 2000; Montaner et al., 2001). A listing of these molecular forms can be found in Table 1, where the amino acid sequences are compared to that of the mammalian form. It should be noted that substitutions have occurred evolutionarily in all locations except those occupied by the pGlu¹ at the N-terminal, the Ser⁴, the Pro⁹, and the glycine-amide at the C-terminal. These data suggest these 4 residues are important for ligand binding

and/or biological activity. Interestingly, mammalian GnRH is active at low concentrations, whereas the remaining vertebrate forms — with the exception of chicken II — show reduced binding affinity and gonadotropin release (Sealfon et al., 1997). The N- and C-terminal domains of GnRH are both involved in receptor binding, while the former also participates in receptor activation and contributes to the biological activity of the decapeptide (Okada et al., 1973). The reason for the conservation of the Ser⁴ residue is unclear since substitutions by a number of different amino acids will retain activity (Sealfon et al., 1997). In contrast, both the Pro⁹ and Gly-amide residues are critical for biological activity and alterations in the C-terminal end of the peptide are known to confer potent agonist or antagonist actions (Karten and Rivier, 1986). In this regard, it is noteworthy that the Pro⁹ residue can be hydroxylated and this peptide can stimulate LH and FSH secretion, albeit less potently than GnRH (Gautron et al., 1995).

With the identification of the various molecular forms of GnRH, it became clear that multiple

species of the peptide co-existed in the brains of reptiles, amphibians, fish, and birds (Fernald and White, 1999). In many cases at least three different isoforms are present and they are typically found in unique locations in brain that include the mid-brain, hypothalamus, and terminal nerve/olfactory system in the telencephalon. Despite this fact, for many years it was thought that only one form of GnRH was present in mammalian brain. However, the musk shrew brain was found to contain a second form (Dellovade et al., 1993; Kasten et al., 1996). Since that time, multiple LHRH species have been identified in rodent (Chen et al., 1998), non-human primate (Lescheid et al., 1997), and human brain (Chen et al., 1998; White et al., 1998a; Yahalom et al., 1999). The hypothalamic form of GnRH is clearly involved in the endocrine regulation of reproduction since a mutation in this gene in mice renders the animal infertile (Cattanach et al., 1977; Mason et al., 1986a) and gene therapy restores reproductive function (Mason et al., 1986b). By contrast, the mid-brain form is thought to play some role in sexual

behavior because injection of high concentrations of mammalian GnRH into this brain region of rats induces lordosis (Sakuma and Pfaff, 1983). Moreover, exposure of female musk shrews to males induces rapid changes in specific populations of GnRH neurons in the midbrain (Dellovade et al., 1993). Currently, both chicken II and the salmon forms of the decapeptide have been found in the midbrains of mammals (Lescheid et al., 1997; Chen et al., 1998; and Yahalom et al., 1999).

The cDNA for GnRH was first cloned in humans by Seeburg and Adelman (1984). Deduction of the primary sequence from the cDNA revealed that the prepro-hormone consisted of 92 amino acids (Fig. 1). At the N-terminal, the signal sequence is composed of 23 amino acids and it is separated from a pro-peptide of 69 amino acids. The GnRH is located at the N-terminal end of the prohormone and is separated by a putative processing site from the remainder of the molecule. The C-terminal portion of the pro-GnRH protein is termed GnRH-associated peptide (GAP). Since some peptide precursors can

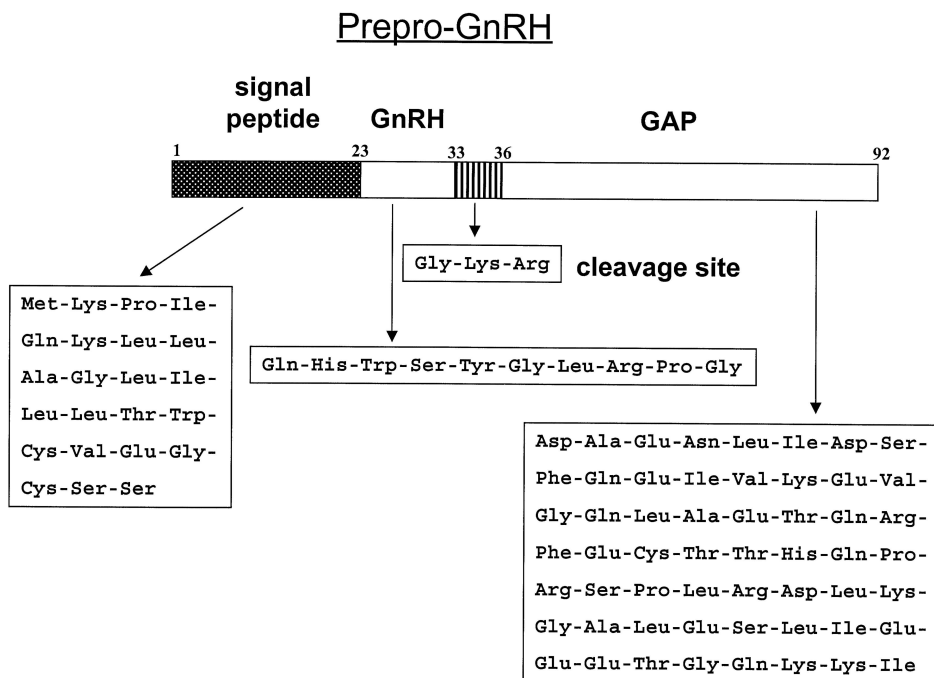


Fig. 1. The deduced structure of the 92 amino acid human prepro-GnRH. The organization of the precursor is shown. The positions of the amino acids that comprise the signal peptide, GnRH, putative cleavage site, and GAP (1-56) are displayed. A three-letter abbreviation is given for the amino acids.

TABLE 2

Comparison of the deduced amino acid sequences of the pro-GnRH from various species ^{a,b}

<i>Mammalian Pro-GnRH</i> ^c	1	10	14	20	30	40	50	60	69										
Human	QHWSYGLRPG	GKR	DAENLIDSFQEIVKEVGQLAETQRFECTTHQPRSPRLDLKGALESLEEETGQKKI																
Rat	QHWSYGLRPG	GKR	NTEHLVDSFQEMGKEEDQMAEPQNFECTVHWPRSPRLDLRGALERLIEEEAGQKKM																
Mouse	QHWSYGLRPG	GKR	NTEHLVESFQEMGKEVDQMAEPQHFECTVHWPRSPRLDLRGALESLEEERQKKM																
<i>Chicken I Pro-GnRH</i> ^d	1	10	14	20	30	40	50	60	69										
Chicken	QHWSYGLQPG	GKR	NAENLVESFQEIANEMESLGEGQKAECPGSYQHPRLSDLKETMASLIEGEARRKEI																
<i>Chicken II Pro-GnRH</i> ^e	1	10	14	20	30	40	50	60	70	80	90	97							
Human	QHWSHGWYPG	GKR	ALSSAQDPQNALRPPGRALDTAAGSPVQTAHGLPSDALAPLDDSMWPWEGRTTAQWSLHRKRHLARTLLTAAREPRPAPPSSNKV																
Rhesus	QHWSHGWYPG	GKR	ALSSAQDPQNALRPP AGSPAQA TYGLPSDALAHLED SMPWEGRTMA W SLRRKRYLAQTLLTAAREPRVPPSSNKV																
<i>Salmon Pro-GnRH</i> ^f	1	10	14	20	30	40	50	59											
Salmon	QHWSYGWLPG	GKR	SVGELEATIKMMDTGCVVALPEETSAAHVSERLRPYDVILKKWMPHK																
<i>Seabream Pro-GnRH</i> ^g	1	10	14	20	30	40	50	60	70										
Seabream	QHWSYGLSPG	GKR	DLDSLSDTLGNIIERFPHVDSPCSVLGCVEEPHVPRMYRMKGFIGSERDIGHRMYKK																
<i>Frog Pro-GnRH</i> ^h	1	10	14	20	30	40	50	60	66										
Frog	QHWSYGLWPG	GKR	EVEGLQESYSEVPNEVSFTDPQHFRSIPQNRISLVREALMNWLEGENTRKKI																
<i>Pejerry Pro-GnRH</i> ⁱ	1	10	14	20	30	40	50	60	70										
Pejerry	QHWSFGLSPG	GKR	ELKYFPNTLENQIRLLNSNTPCSDLHLEESSLAKIYRIKGLLGSVTEAKNGYRTYK																
<i>Lamprey I Pro-GnRH</i> ^j	1	10	14	20	30	40	50	60	63										
Lamprey	QHYSLEWKPG	GKR	DLEQELEPPSNAFECDGPECAFSRVPNTKLIRELASYLSQRNYDRKGALK																
<i>Catfish Pro-GnRH</i> ^k	1	10	14	20	30	40	50	59											
Catfish	QHWSHGLNPG	GKR	AVMQESAEEIPRRSGYLCDYVAVSPRNKPFRLKDLLTPVAGREIEE																

^a The amino acid sequences are provided as single letter abbreviations; differences between subspecies are only highlighted for mammalian pro-GnRH and chicken II pro-GnRH.^b The N-terminal containing the GnRH moiety is separated by a space from a putative processing site and the GnRH-associated peptide; the numbers over the peptide sequence represent the positions of the amino acids.^c Mammalian pro-GnRH: human – Seeburg and Adelman, 1984; rat – Adelman et al., 1986; mouse – Mason et al., 1986a.^d Chicken I pro-GnRH: chicken – Dunn et al., 1993.^e Chicken II pro-GnRH: human – White et al., 1998a; rhesus monkey – White et al., 1998b.^f Salmon pro-GnRH: salmon – Klungland et al., 1992.^g Seabream pro-GnRH: seabream – Gothilf et al., 1995.^h Frog pro-GnRH: frog – Yoo et al., 2000.ⁱ Pejerry pro-GnRH: pejerry – Okubo et al., 2000.^j Lamprey I pro-GnRH: lamprey – Suzuki et al., 2000.^k Catfish pro-GnRH: catfish.

be processed into a variety of different bioactive peptides, several investigators have studied the activity of GAP in pituitary cultures. In the initial report, a recombinant GAP protein was biosynthesized in bacteria, purified, and added to primary rat pituitary cell cultures (Nikolics et al., 1985). At 10 μ M the recombinant GAP influenced secretion of LH, FSH, and prolactin, but it exerted no effects on adrenocorticotropin or growth hormone release. GAP stimulated LH release, albeit less effectively than GnRH while it was equipotent with the decapeptide for FSH secretion. By comparison, GAP potently inhibited prolactin secretion and this inhibition was not synergistic with dopamine. It was noted that during the process of raising GAP antisera in rabbits, serum prolactin levels became depressed. Due to these collective effects, GAP was described as a prolactin-inhibiting factor. Since that time, GAP has been shown to be co-released with GnRH from rat median eminence fragments in vitro (Valença et al., 1988; Wetsel and Negro-Vilar, 1989) and into the ovine hypophyseal portal circulation in vivo (Clarke et al., 1987). Additionally, there is some evidence that some GAP-(1–56) may be processed into smaller fragments in rat hypothalamus (Ackland et al., 1988; Wetsel et al., 1988). GAP has been reported to decrease the intracellular levels of $[Ca^{2+}]$ from human prolactin-secreting adenoma cells, rat pituitary GH3 cells, and primary rat pituitary cells (Vacher et al., 1991). It has also been observed to reduce cAMP levels in GH3 cells (van Chuoi et al., 1993).

In comparison to secretion and peptide processing studies, the physiological role of GAP is controversial. Human recombinant GAP-(1–56) was initially reported to inhibit prolactin secretion from rat pituitary cells (Nikolics et al., 1985). On the other hand, the synthetic peptide can stimulate prolactin secretion from tilapia pituitary (Planas et al., 1990). Interestingly, different domains of the GAP molecule appear to possess differing biological activities. In tilapia, the stimulatory effect seemed to reside within the GAP-(28–36) and GAP-(51–66) fragments, whereas GAP-(38–49) was inhibitory. In rat pituitary cell cultures, various synthetic GAP fragments have been observed to influence gonadotropin (Milton et al., 1986) and prolactin release (Wormald et al., 1989). Additional studies conducted

with rats in vivo have shown GAP-(1–56) can inhibit prolactin release, while the effects on LH and FSH secretion are very limited (Yu et al., 1988). On the other hand, GAP-(1–13) and GAP-(1–23) may have some gonadotropin-releasing activities (Yu et al., 1989). By contrast, in ovariectomized ewes, GAP-(1–56) exerts no effects on secretion of LH, FSH, or prolactin (Thomas et al., 1988a). A similar failure of GAP to inhibit prolactin release in rats (Chandrashekar et al., 1988) and cells from human pituitary adenomas has also been noted (Ishibashi et al., 1987). In summary, our knowledge on the physiology of GAP remains obscure.

Irrespective of a possible physiological role for GAP, it should be noted that during protein synthesis the ribosome encloses approximately 30–35 amino acids of the nascent protein (Lewin, 1996). Since the mammalian prepro-GnRH contains 92 amino acids and the first 23 amino acids contain the signal sequence, one purpose of GAP-(1–56) may be to serve as a linker to the decapeptide so that the pro-GnRH can be routed out of the endoplasmic reticulum and into the secretory pathway.

Although 15 different molecular forms for GnRH have been identified at the peptide level, only nine of the complementary DNAs for these forms have been cloned (Table 2; Seeburg and Adelman, 1984; Adelman et al., 1986; Mason et al., 1986a; Klungland et al., 1992; Dunn et al., 1993; Bogerd et al., 1994; Gothilf et al., 1995; White et al., 1998a,b; Okubo et al., 2000; Suzuki et al., 2000; Yoo et al., 2000). Examination of the deduced amino acid sequences reveals that in all cases, GnRH is located at the N-terminal end of the precursor and it is separated by a putative processing site from GAP. In all of the deduced sequences for the pro-GnRH, the least conservation of amino acids occurs within the GAP portion of the molecule. The rat and mouse forms of GAP in the mammalian GnRH are only 70% homologous to the human GAP-(1–56) sequence. For the chicken II GAP proteins, divergence is even greater among the deduced sequences for the human and rhesus monkey. The divergence of the GAP sequence between even closely related species suggests that, if this peptide has bioactivity, it may be different among these species.

Scheme for Pro-GnRH processing

Inspection of the deduced amino acid sequences from the nine complementary pro-GnRH forms demonstrates several points. First, all pro-GnRH proteins contain GnRH at their N-termini and the peptide is separated by a putative processing site from GAP. The organization and spatial arrangement of these sequences suggests that the enzymes that process the pro-GnRH in a given cell may be similar across all species. Second, excision of the GnRH peptide from the precursor must be performed by an endopeptidase. Since most peptide precursors appear to be processed at monobasic or dibasic residues (Douglass et al., 1984), the endopeptidase could cleave the pro-GnRH after the Lys¹² or Arg¹³ residues. Third, because the decapeptide does not contain these basic residues at its C-terminal, there must be some exopeptidase that can remove them. Fourth, an examination of the decapeptide shows that the first amino acid of the GnRH is always Gln¹. Since the fully processed GnRH contains a pGlu¹ at its N-terminal, some enzyme must be present to process the Gln¹ to pGlu¹. Fifth, because the C-terminal of GnRH is amidated, some enzyme must be responsible for converting the Gly¹¹ to an amide. Finally, there is some evidence that the Pro⁹ residue can be hydroxylated to produce [Hyp⁹]GnRH. A depiction of this processing scheme can be found in Fig. 2.

Prohormone convertases

With the cloning of many peptide hormone and neuropeptide cDNAs and following the deduction of their amino acids sequences, it became clear that bioactive peptides are first biosynthesized as precursor proteins. Typically, the N- and/or C-terminals of the peptides are bounded or separated from the remainder of the precursor by monobasic or dibasic residues (Douglass et al., 1984). Since trypsin can cleave proteins at these residues, investigators searched for many years for native trypsin-like enzymes that could process pro-peptides. The first breakthrough in the discovery of prohormone processing enzymes came with the identification of kexin (an enzyme encoded by the *Kex2* gene); this enzyme was responsible processing α -mating factor in *Saccharomyces cerevisiae* (Julius et al., 1984).

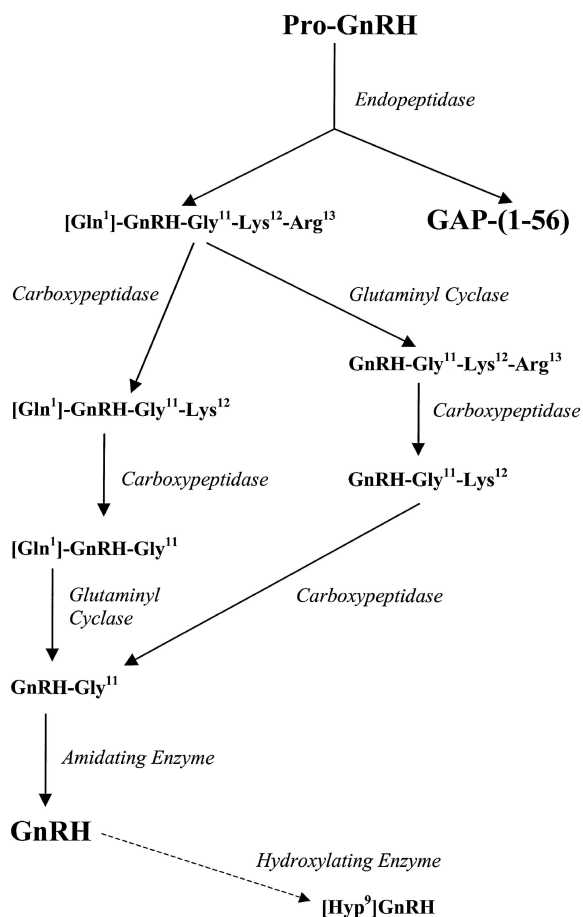


Fig. 2. A processing scheme for the pro-GnRH to bioactive GnRH. The prohormone is processed by an endopeptidase to yield a GnRH intermediate and GAP-(1-56). The intermediate has several fates. The C-terminal basic amino acids can be removed by a carboxypeptidase, the N-terminal glutamine can be converted to a pyroglutamate by a glutaminyl cyclase, and the C-terminal glycine can donate its amide group through the action of an amidating enzyme to produce GnRH. Alternatively, the N-terminal of the intermediate can be processed to the pyroglutamate, the C-terminal basic amino acids removed, and the GnRH can be amidated. There is also some evidence that the proline residue of GnRH can be hydroxylated by some hydroxylating enzyme and this step is placed at the end of the processing cascade only for convenience. At this time, it is unclear at which step(s) the hydroxylation reaction occurs. The N- and C-terminal amino acids of the intermediate are designated by three-letter abbreviations; the numbers signify the amino acid positions in the intermediate.

Subsequent studies demonstrated the ability of kexin to correctly process exogenously expressed mammalian pro-hormones in yeast (Thim et al., 1986)

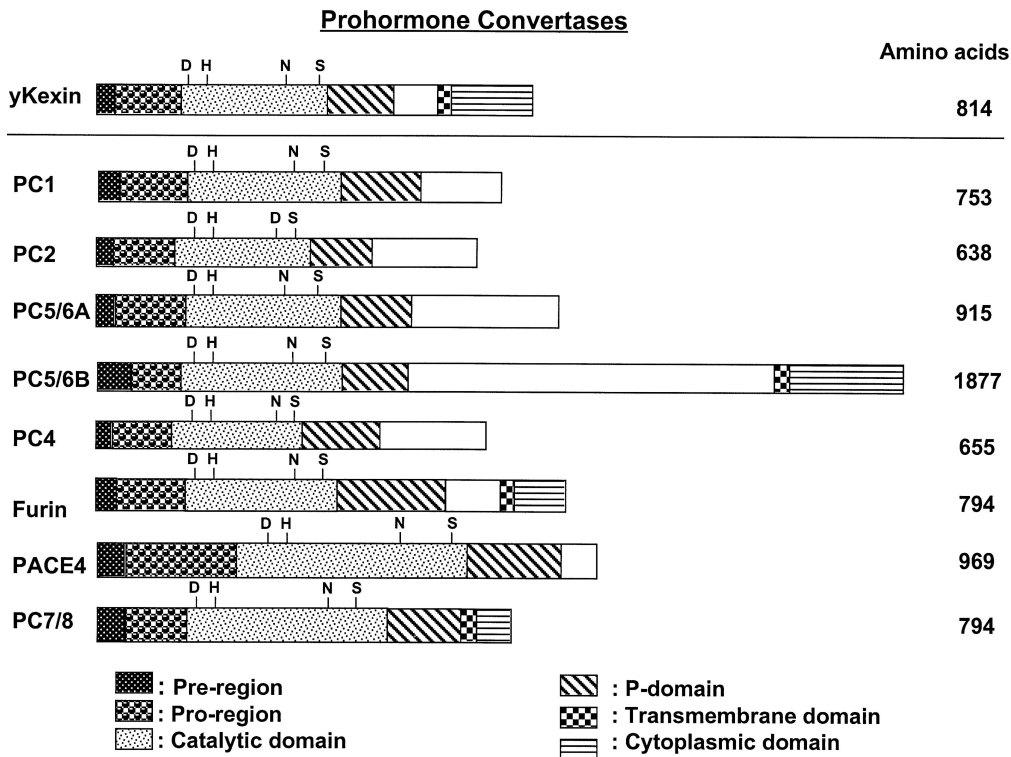


Fig. 3. A depiction of the yeast Kex2 enzyme and the mammalian prohormone convertases. The organization of the enzymes is depicted in the diagram. The catalytic sites on the endopeptidases are identified by the single amino acid designations. The significance of the shading for each enzyme can be found at the bottom of the figure. The pre-region contains the signal peptide. The P-domain is important for the correct folding of the convertases in the endoplasmic reticulum. The open bars refer to 'spacer' regions. The numbers of amino acids that comprise the proenzymes are designated to the right of each enzyme.

and in various mammalian cell lines (Thomas et al., 1988b). Later, in the course of cloning the *c-fes/fps* oncogene, it was noted that a potential open reading frame for another gene was present upstream of the oncogene and that its sequence bore some homology to that of Kex2 (Roebroek et al., 1986). This putative protein was designated *fes/fps* upstream region or furin. To date, eight members of the family of the prohormone convertases (PCs) have been identified (Fig. 3) and these include furin (also termed Paired Amino Acid Converting Enzyme or PACE), PC1/3 (Seidah et al., 1990, 1991), PC2 (Seidah et al., 1990; Smeekens and Steiner, 1990), PC4 (Nakayama et al., 1992; Seidah et al., 1992), PACE4 (Kiefer et al., 1991), PC5/6 (Lusson et al., 1993; Nakagawa et al., 1993a,b), PC7/8 (Bruzaniti et al., 1996; Seidah et al., 1996), and Ski-1 (Seidah et al., 1999a). Since the latter enzyme has been shown to process growth

factors, receptors, and viral glycoproteins, it will not be discussed further.

The PCs differ in their tissue distributions and intracellular locations (Steiner, 1998; Seidah and Chrétien, 1999). Furin and PC7/8 are ubiquitous in their expression (Schalken et al., 1987; Bruzaniti et al., 1996; Seidah et al., 1996), while both PC1 and PC2 are localized exclusively in neural and endocrine tissues (Seidah et al., 1990, 1991; Smeekens and Steiner, 1990). Within the central nervous system, PC2 is more widely expressed than PC1 with both convertases being expressed in the hypothalamus (Seidah et al., 1991; Schäfer et al., 1993). PC4 expression has been detected exclusively in reproductive tissues. More specifically, it is located in round spermatids and spermatozoa (Seidah et al., 1992) and in macrophage-like cells of the ovary (Tadros et al., 2001). PACE4 and PC5/6 reside in

both endocrine and non-endocrine cells (Kiefer et al., 1991; Lusson et al., 1993). PACE4 is highly expressed in the pituitary and chondrocytes. PC5/6 exists in two splice forms; PC5/6A is distributed more widely than PC5/6B and it is expressed in both endocrine and non-endocrine cells (Nakagawa et al., 1993a,b; Dong et al., 1995). In rat brain, PC5/6A immunoreactivity is more restricted in comparison to that for PC1/3 and PC2. It is present throughout the forebrain, as well as in the diagonal band of Broca and hypothalamus. As is apparent from the pattern of expression, each convertase has its characteristic tissue distribution. However, there is an overlap in the expression of two or more convertases in several tissues. This finding implies that there may be considerable redundancy of function among certain of the enzymes.

The PCs are an evolutionarily conserved family of calcium-dependent proteases that are related to bacterial serine proteases of the subtilisin family of convertases (Steiner, 1998; Seidah and Chrétien, 1999). Due to this relationship and because the PCs are also associated with Kex2, the PC family of enzymes has also been called subtilisin-like pro-protein convertases or subtilisin/Kex2 family of enzymes. All of these enzymes are synthesized as precursors that contain a pre-domain, a pro-domain, a catalytic domain, a P-domain, and an enzyme-specific C-terminal domain (see Fig. 3). The pre-region contains the signal peptide. The pro-region serves as an intra-molecular chaperone, as well as a transient intra-molecular inhibitor of enzymatic activity. The conserved catalytic domain contains a catalytic triad consisting of Asp, His and Ser residues that are involved in oxyanion stabilization and are important in catalysis. The catalytic domain for PC2 contains an Asn instead of the Asp residue. The P-domain is important for the correct folding of the convertases in the endoplasmic reticulum. The C-terminal regions of these enzymes are relatively variable (Bergeron et al., 2000). While furin, PACE4, and PC5/6B contain cysteine-rich regions, PC1 and PC2 have amphipathic helices, and PC7/8 contains a serine-threonine-rich region in the C-terminal. Furin, PACE4E, PC5/6B, and PC7/8 also have transmembrane domains in this region.

As noted above, the PCs are first biosynthesized as inactive precursors and they must undergo prote-

olytic processing to achieve full enzymatic activity (Bergeron et al., 2000). The pro-segment of the enzyme can be removed by an autocatalytic reaction. This removal plays a role in enzymatic activation and it is necessary for sorting the convertase into the appropriate secretory pathway. Besides pro-region retardation of enzymatic activity, there are also endogenous proteins that can inhibit PC1/3 and PC2. A specific endogenous inhibitor for PC1/3 has been recently identified as a polypeptide termed pro-SAAS (Fricker et al., 2000). Pro-SAAS only inhibits PC1/3 and none of the other PC members (Fricker et al., 2000; Qian et al., 2000). Pro-SAAS is expressed in all regions where PC/3 resides, as well as in additional regions, suggesting that it may have some undisclosed functions (Feng et al., 2001; Lanoue and Day, 2001; Sayah et al., 2001). Similar to PC1/3, PC2 also interacts with an additional protein, 7B2 (Braks and Martens, 1994; Lindberg et al., 1995; Zhu and Lindberg, 1995). The 7B2 is a neural and endocrine polypeptide that binds to inactive pro-PC2. It facilitates the transport of the convertase from the endoplasmic reticulum to later compartments in the secretory pathway where PC2 can undergo proteolytic maturation. The C-terminal peptide of 7B2 can inhibit PC2. All cells that express PC2 also express 7B2. However, the converse is not true (Seidah et al., 1999b), suggesting that 7B2 might have some additional roles in the neuronal and endocrine cells. Mice lacking the 7B2 gene have impaired processing of pro-glucagon, pro-insulin, pro-enkephalin, and pro-opiomelanocortin and do not have any detectable PC2 activity (Westphal et al., 1999). These mutants display Cushing's syndrome and die within 9 weeks of age.

Research with a number of different systems has clearly established a role for the PCs in processing precursor hormones and neuropeptides. Since endoproteolysis represents the first step in pro-GnRH processing (see Fig. 2), we wanted to determine whether any of these enzymes could process the prohormone. At that time, only furin, PC1/3 and PC2 had been cloned. Since the immortalized hypothalamic LHRH neuronal cell lines can process the precursor (Wetsel et al., 1991), we selected these neurons for study. Results from Northern blots revealed that the GT1 cells contained transcripts for furin and PC2 (Wetsel et al., 1995). No transcripts

were evident for PC1/3 even when poly(A)⁺ RNA was used. These results are consistent with an in-situ hybridization study in which PC2 transcripts were found in LHRH neurons in rat hypothalamus (Voigt et al., 1996). To determine whether PC2 could process the pro-GnRH, vaccinia virus recombinants of the prohormone, furin, PC1/3, and PC2 were used in studies conducted in collaboration with Dr. Gary Thomas at the Oregon Health Sciences University in Portland Oregon. Preliminary results conducted in BSC-40 (kidney) cells demonstrate that furin, PC1/3 and PC2 can all process the pro-GnRH. Whereas furin and PC1/3 are very inefficient in converting the pro-hormone to GnRH and GAP-(1–56), PC2 is efficient in this process.

Another way to evaluate the role of the PCs in processing the pro-GnRH is through the use of mutants where the genes for these enzymes have been selectively deleted. Since furin (Roebroek et al., 1998) and PACE4 (Constam and Robertson, 2000) null mice exhibit embryonic lethality during mid-gestation, the effects of these enzymes on pro-GnRH processing and reproduction cannot be easily evaluated in vivo. By comparison, PC4 expression is exclusively gonadal and is not expressed in brain (Seidah et al., 1992). PC4 null males are infertile and there is reduced fertility in females (Mbikay et al., 1997). There are no published reports on PC5/6 or PC7/8 null mice.

Preliminary analyses of PC1/3 null mutants indicate that these animals die in utero (see Seidah and Chrétien, 1999). Interestingly, an adult human female with compound heterozygous mutations for PC1/3 (a missense mutation in one allele and a splicing mutation in the other allele) has been identified (O'Rahilly et al., 1995; Jackson et al., 1997). The pro-PC1/3 derived from the missense mutation would be expected to be retained in the endoplasmic reticulum, while the product derived from aberrant splicing at exon 5 would result in a frameshift with premature termination of translation in the catalytic domain. This patient had childhood obesity, hypogonadotropic hypogonadism, hypocortisolism, and elevated levels of circulating pro-insulin. The discrepancy in viability of the human patient and the PC1/3 knockout mice may be attributed to the possibility that there may be residual enzyme activity in the human patient despite mutations in both alle-

les. Alternatively, PC1/3 may have acquired slightly different functions in humans.

PC2 null mice have been described (Furuta et al., 1997, 2001). These mutants exhibit β -cell defects, mild hypoglycemia, and a small decrease in the rate of postnatal growth. The animals display a deficiency, but not complete blockade, in pro-insulin, pro-glucagon, and pro-somatostatin processing. The PC2 null mice also demonstrate an impairment in processing pro-enkephalin (Johanning et al., 1998), pro-cholecystokinin (Vishnuvardhan et al., 2000), pro-dynorphin (Berman et al., 2000), pro-opiomelanocortin and pro-orphanin FQ/nociceptin (Allen et al., 2001). Preliminary results from our laboratory have demonstrated that PC2 null mice exhibit mild reproductive dysfunction as exemplified by delayed onset of puberty, irregular estrous cycles, and impaired ability of the dams to nurture their offspring. Additional results suggest that there may be some deficiency in pro-GnRH processing, but this deficit is not complete. Hence, these data indicate that some additional convertase may be compensating for the loss of PC2. Recent preliminary findings from our lab suggest that PC5/6A may be a compensating enzyme.

Carboxypeptidase

Following endoproteolytic cleavage of a peptide precursor by prohormone convertase(s), the C-terminal basic amino acids must be removed. Carboxypeptidases hydrolyze amino acids from the C-terminal of peptides and proteins. A family of 25 carboxypeptidases have been identified and their mechanisms of cleavage use an active site at serine or cysteine residues, or at zinc (Reznik and Fricker, 2001). From the enzymes comprising the latter group, approximately 13 different metallocarboxypeptidase genes have been cloned (Fig. 4). A carboxypeptidase that is expressed almost exclusively in neural and endocrine tissues is carboxypeptidase E (CPE; Fricker et al., 1986; Rodriguez et al., 1989; Fricker, 1991; Schäfer et al., 1993; Zheng et al., 1994). This carboxypeptidase was first identified as enkephalin convertase (Fricker and Snyder, 1972) and it has also been termed carboxypeptidase H (or EC3.4.17.10; Parkinson, 1990; Fricker, 1998). While the enzyme shows broad peptide specificity, it is highly specific in its

Carboxypeptidases

Amino acids

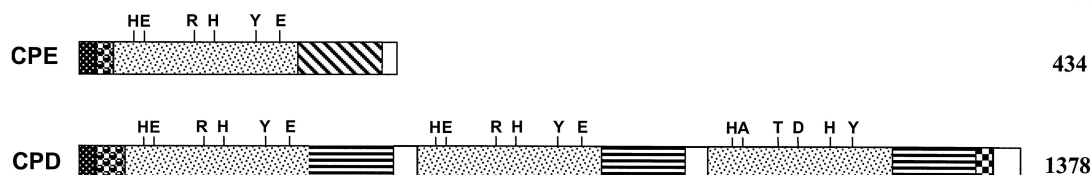


Fig. 4. A diagram of carboxypeptidases E and D. The organization of the enzymes is depicted in the diagram (see Fig. 3 for details). The sites on the carboxypeptidases (e.g., H, E, and H bind Zn^{2+} , R and Y bind the substrate, and with the E all sites participate in catalysis) are identified by the single amino acid designations. The significance of the shading for each enzyme can be found at the bottom of Figure 3. The open bars refer to 'spacer' regions, whereas the region with the dark horizontal bars represents transthyretin-like domains. The numbers of amino acids that comprise the proenzymes are designated to the right of each enzyme.

removal of C-terminal basic amino acids. Excision of arginine and lysine residues occurs more readily than histidine with this enzyme (Fricker and Snyder, 1972; Smyth et al., 1989). CPE is biosynthesized as a pro-enzyme and this precursor possesses full activity (Parkinson, 1990). The N-terminal extension of the enzyme is removed in a post-Golgi compartment or secretory vesicle (Song and Fricker, 1995a) to yield a glycosylated metalloenzyme of approximately 55,000 kDa in size. The enzyme displays optimal activity between pH 5–5.5 and is inactive at neutral pH. Since the secretory vesicle has an acidic pH and because endoproteolysis of many peptides occurs within the *trans*-Golgi network and secretory vesicle (Douglass et al., 1984), juxtaposition of CPE with peptide intermediates in the late secretory pathway is optimal for its activity (Fricker, 1998). Within the regulated secretory pathway, CPE is present both as soluble and membrane-bound forms that arise through differential posttranslational processing (Fricker et al., 1990). Despite this fact, the catalytic activities of these forms are similar.

In early studies of pro-GnRH processing in rat hypothalamus, we proposed that a carboxypeptidase may be involved in converting pro-GnRH intermediates to GnRH (see Fig. 2; Wetsel et al., 1988). Later, we showed in the immortalized GnRH cell lines that the prohormone was cleaved between the Arg¹³ and Asn¹⁴ residues to produce Gln¹-GnRH-Gly¹¹Lys¹²Arg¹³ and GAP-(1–56) (Wetsel et al., 1991). Since we also found evidence that the Arg¹³ and Lys¹² residues could be removed from the GnRH intermediate and because CPE was expressed in brain, we postulated that this enzyme

was responsible for removing these C-terminal basic amino acids from the precursor. In subsequent work, we showed that the immortalized GnRH cells and the brain regions (e.g., preoptic area and hypothalamus) that contains these neurons possessed transcripts for CPE (Wetsel et al., 1995). To demonstrate that CPE could actually process these C-terminally extended peptides, we conducted two different experiments. First, we incubated in vitro synthetic GnRH-Gly¹¹Lys¹²Arg¹³ or GnRH-Gly¹¹Lys¹² with purified recombinant baculovirus-derived CPE. Both peptides were processed to GnRH-Gly¹¹ by the enzyme. Second, we used vaccinia viral recombinants of CPE to examine processing of the pro-GnRH in mammalian cells. In this case, recombinant cDNAs for pro-GnRH, PC2, and CPE were expressed in kidney cells that do not normally express any of these genes. The PC2 was expressed so that the prohormone could be endoproteolytically cleaved to an intermediate with C-terminally extended basic amino acids. CPE was found to remove the Arg¹³ and Lys¹² residues from this intermediate. Collectively, these findings clearly established a role for CPE in pro-GnRH processing, however, an in vivo role in mammals remained to be determined.

More than 10 years ago, a spontaneous autosomal recessive mutation was identified in a colony of mice at Jackson Laboratories (Coleman and Eichler, 1990). These animals were found to be obese, diabetic, and infertile and they were called *fat/fat* mice. Since the animals begin to develop obesity after puberty, they have been used as an animal model for adult-onset obesity for many years (Leiter and Herberg, 1997). Subsequent mapping studies have

revealed that the locus of the *fat* mutation is on chromosome 8 and that it is close to the murine *Cpe* gene (Prochazka et al., 1991). Additional experiments have demonstrated that the CPE gene in *fat/fat* mice is mutated at a single nucleotide that results in a single amino acid change, Ser²⁰²Pro. Due to identification of the mutation in these mice, the nomenclature for these animals was changed to *Cpe^{fat}*. Interestingly, CPE mRNA levels are similar in wild type (WT), heterozygous, and homozygous mice. Despite this fact, the CPE protein is undetectable in the mutants because the mutated enzyme is unstable and is rapidly degraded in the endoplasmic reticulum (Naggert et al., 1995; Varlamov et al., 1996). As a result, CPE activity in the pancreas and pituitaries of the homozygotes is very low and is reduced by at least 20-fold from that of the WT animals.

Since we had found that CPE transcripts were present in the immortalized GnRH neurons and in brain regions where GnRH neurons reside in situ (Wetsel et al., 1995), we postulated that the infertility of the *Cpe^{fat}* mice might be due to a processing deficit for pro-GnRH. To this purpose, we dissected hypothalami from WT and *Cpe^{fat/fat}* animals, separated the materials by high pressure liquid chromatography, and screened the fractions with an antiserum that recognized all GnRH intermediate peptides. Our analysis revealed that the homozygotes were deficient in processing the pro-GnRH such that there was a substantial increase in the quantities of GnRH–Gly¹¹Lys¹²Arg¹³ and GnRH–Gly¹¹Lys¹². Some fully processed GnRH was detected in mutant hypothalamus, however, it was reduced by at least 75%. These data clearly show that CPE plays a major role in vivo in processing the pro-GnRH.

Since the *Cpe^{fat}* mouse is deficient in pro-insulin processing (Naggert et al., 1995), various investigators have tried to determine whether human diabetic patients may also have mutations in *Cpe*. While a number of different polymorphisms in the *Cpe* gene have been described in these patients (Utsunomiya et al., 1998), only one has been associated with diabetes (Chen et al., 2001). In this case, a single nucleotide alteration in C⁸⁴⁷T renders a codon change of Arg²⁸³Trp. Since this alteration reduces the efficiency of CPE to cleave its substrates and because the enzyme is unstable at elevated temperatures, this

polymorphism may predispose homozygous patients to hyperproinsulinemia and diabetes. At present, the reproductive status of the patients is unknown.

In the genetic characterization of the *Cpe^{fat}* mouse and in our own analysis, it was observed that while conversion of pro-insulin in pancreas or pro-GnRH in hypothalamus was perturbed, a small amount of biologically active peptide was still present. In addition, a very small amount of CPE-like activity was detected. These findings suggest that there might be some other carboxypeptidase that could convert the intermediates to bioactive peptides.

Although the family of metallocarboxypeptidases has significantly expanded over the past five years (Reznik and Fricker, 2001), there is one member of this family that may be able to process peptide precursors and it may account for the partial rescue of the *Cpe^{fat}* mouse. Some years ago, a surface protein in ducks that binds hepatitis B viral particles was cloned and it was termed gp180 (Kuroki et al., 1994). Independently, a carboxypeptidase activity different from that for CPE was purified from bovine pituitary and this enzyme was found to remove basic amino acids from the C-terminal of peptides (Song and Fricker, 1995b). This carboxypeptidase was called CPD (EC 3.4.17.22). Sequencing and subsequent cloning results indicated that gp180 and CPD were the same protein (Kuroki et al., 1995; Song and Fricker, 1995b; Tan et al., 1997; Xin et al., 1997). CPD is approximately 180 kDa in size and it contains three tandem-repeat carboxypeptidase-like domains (see Fig. 4); however, only the first two domains are catalytically active (Song and Fricker, 1996). The first domain is maximally active at pH 6.3–7.5 and it prefers C-terminal arginine substrates, whereas the second domain is active at pH 5.0–6.5 and it prefers C-terminal lysine (Novikova et al., 1999). Recently, the second domain of CPD has been crystallized (Gomis-Rüth et al., 1999). Since CPD and CPE have high homologies in the catalytic domains, it has been proposed that similar functional groups are responsible for substrate binding and catalysis (Aloy et al., 2001). CPD is a glycosylated enzyme, it exists in both soluble and particulate forms, and it is widely distributed in many different tissues (Song and Fricker, 1996). While CPD is highly expressed in brain, pituitary and adrenal gland, expression is even higher than CPE in certain brain regions (Song

and Fricker, 1996; Dong et al., 1999). Within cells the enzyme is found in the *trans*-Golgi network and immature secretory granules, however, it is absent from mature granules (Varlamov et al., 1999). At the present time it is not known whether GnRH neurons contain CPD. Nonetheless, given its location within other cells, CPD would be expected to be greatly restricted in its ability to process pro-GnRH intermediate peptides.

Amidating enzyme

An initial problem in the identification of GnRH involved its sequencing. Traditional sequencing methods were unable to resolve the C-terminal residue of the peptide. Tritiation (Matsuo et al., 1971) or hydrazinolysis (Burgus et al., 1972) were finally able to show that the C-terminal of GnRH contained a glycine amide. Since this residue is not a naturally occurring amino acid, it must be produced through the action of some proteolytic enzyme. An enzyme activity that could produce C-terminal amides from glycine was later identified (Bradbury et al., 1982) and it was shown to require molecular oxygen, copper, and ascorbic acid for full activity (Eipper et

al., 1983). This enzyme was termed peptidylglycine α -amidating monooxygenase (PAM). The cDNA for an amidating enzyme was first cloned from frog skin (Mizuno et al., 1987) and this was rapidly followed by isolation of a PAM cDNA from bovine neurointermediate pituitary (Eipper et al., 1987). PAM was also cloned from rats and it was found to consist of a single gene, to contain at least 27 exons, to span a region of more than 160 kb, and to be located on human chromosome 5 (Ouafik et al., 1992). Alternative splicing produces at least 7 different rat PAM proteins that range in size from approximately 35,000–108,000 molecular weight (see Fig. 5; Eipper et al., 1992a,b). These and other data revealed that PAM is a bifunctional enzyme (Kato et al., 1990; Katopodis et al., 1990, 1991; Perkins et al., 1990; Takahashi et al., 1990). The large PAM precursor protein or PAM-1 contains an N-terminal signal sequence, a short pro sequence, a catalytic region consisting of peptidylglycine α -hydroxylating monooxygenase (PHM or EC 1.14.17.3), a connecting domain, a second catalytic region consisting of peptidyl- α -hydroxyglycine α -amidating lyase (PAL or EC 4.3.2.5), a transmembrane domain, and a short C-terminal (see Fig. 5; Eipper et al., 1992a). PAM-

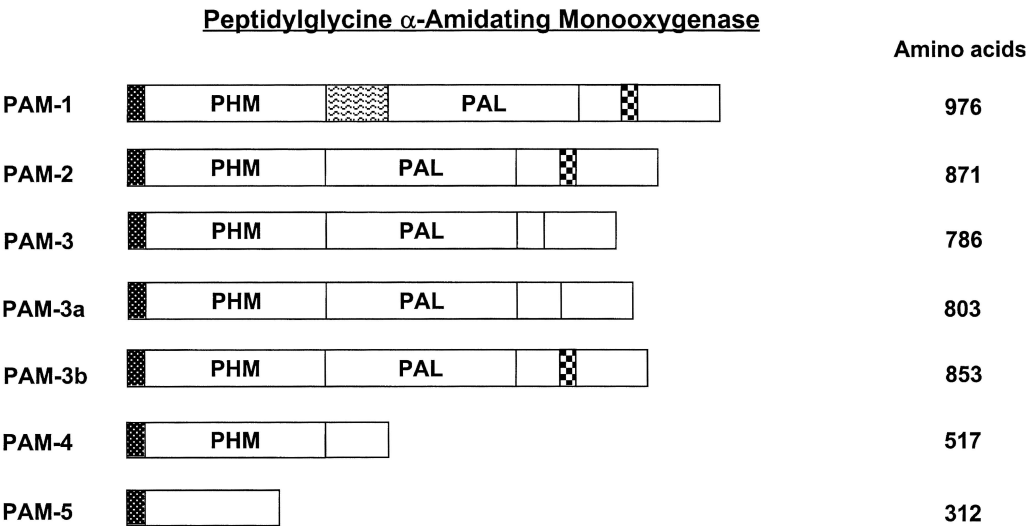


Fig. 5. Depiction of the 7 forms derived from peptidylglycine α -amidating monooxygenase (PAM). These enzymes are synthesized as proenzymes. The catalytic peptidylglycine α -hydroxylating monooxygenase (PHM) and peptidyl- α -hydroxyglycine α -amidating lyase (PAL) domains are shown. The PAM-1, -2, and -3b contain transmembrane domains, while the PAM-3, -3a, -4, and -5 are soluble proteins. The significance of the shading for each enzyme can be found at the bottom of Figure 3. The shading in PAM-1 between the PHM and PAL domains is encoded by an exon whose product is absent in all other PAM forms. The numbers of amino acids that comprise these enzymes are listed to the right.

1, -2, -3, -3a, and -3b all contain both PHM and PAL catalytic regions, while only PAM-1, -2, and -3b contain the transmembrane domains (Stoffers et al., 1989, 1991; Eipper et al., 1992a). PAM-4 has the PHM domain. PAM-5 is shorter than PAM-4 and is not thought to possess enzymatic activity. Its function is currently unknown.

PHM catalyzes the first step of the amidation reaction where the C-terminal glycine is hydroxylated, while PAL is responsible for the second reaction where the glyoxylate moiety is removed from the C-terminal of the intermediate and the amide group from the original hydroxyglycine is donated to the peptide (Bradbury et al., 1982; Eipper et al., 1983; Kato et al., 1990; Katopodis et al., 1990, 1991; Perkins et al., 1990). In mammals the PHM and PAL may be expressed within the same precursor, while in other organisms they may be expressed independently of each other (Prigge et al., 2000). Interestingly, in *Drosophila* deletion of the PHM gene is embryonically lethal (Kolhekar et al., 1997).

In a survey of the various bioactive peptides in tissues, all 20 amino acid residues at the C-terminal have the ability to be amidated. Besides peptides, purified PHM can catalyze O-dealkylation, N-dealkylation, and sulfoxidation reactions of non-peptide substrates (Katopodis and May, 1990). Recombinant PAM can convert fatty acyl glycines to fatty acyl amides such as oleamide that has been implicated in sleep (Cravatt et al., 1995). It can also convert nicotinuric acid to nicotinamide (Merkler et al., 1999). Since PAM is the only amidating enzyme that has been identified so far, it is presumed to be responsible for these various reactions (Eipper et al., 1992a; Prigge et al., 2000). Despite this fact, glycine-extended peptides for gastrin, adrenomedullin, and oxytocin have been found in blood (Amico and Hempel, 1990; Kaise et al., 1995; Kitamura et al., 1998). Additionally, it is recognized that gastrin-Gly¹⁸ can bind to a receptor that is distinct from that for gastrin (Kaise et al., 1995; Dockray et al., 1996). With respect to GnRH, glycine-extended peptides have been isolated from hypothalamus and the immortalized hypothalamic GnRH neurons, however, the levels are very low (Wetsel et al., 1991). Despite this fact, PAM is expressed in the GT1 cells and in the brain region where GnRH perikarya are located (Wetsel, 1995; Wetsel et al., 1995). In addition, since

chronic treatment with diethylthiocarbamate (an inhibitor of PAM) leads to an increase in LHRH-Gly¹¹-like immunoreactivity in the hypothalamus (Kumar et al., 1994), PAM is probably intimately involved in the *in vivo* processing of the pro-GnRH protein to GnRH.

One substance that has been shown to act directly on GnRH neurons (GT1 cells) and to regulate pro-GnRH processing is basic fibroblast growth factor (Wetsel et al., 1996). Although the immortalized cells contain receptors for this growth factor, no effects on GnRH secretion were discerned (Voigt et al., 1996). This was a surprising result because in most systems, basic fibroblast growth factor stimulates protein kinase C activation. This kinase is known to be a strong stimulator of GnRH secretion from median eminence tissue fragments *in vitro* (Valença et al., 1988) and from the immortalized GnRH neurons (Bruder et al., 1992; Wetsel et al., 1993). Analysis of the media from stimulated cells reveals, however, that the conversion of GnRH-Gly¹¹ to GnRH was partially blocked so that the glycine-extended peptide was one of the predominant forms that was secreted (Wetsel et al., 1996). Presently, the physiological significance of the release of this peptide is unknown because it does not stimulate the secretion of LH or FSH from primary rat anterior pituitary cell cultures.

Glutaminyl cyclase

In the initial isolation and sequencing of GnRH, there was difficulty in determining the N-terminal amino acid. Mass spectrometry was required to identify the pyroglutamate in this position (Matsuo et al., 1971; Burgus et al., 1972). With the cloning of the human pro-GnRH, the deduced sequence revealed that the N-terminal amino acid for GnRH was a glutamine (Seeburg and Adelman, 1984). Since a pyroglutamyl residue is not a commonly occurring amino acid and because N-terminal glutamine is unstable, it was thought that this residue arose spontaneously under physiological conditions. Later, two groups independently isolated an enzyme that could convert glutaminyl into pyroglutaminyl peptides (Busby et al., 1987; Fischer and Spiess, 1987). The subcellular distribution of glutaminyl cyclase (QC) and PAM are virtually identical. QC activity

Glutaminyl Cyclase

Amino acids

QC



361

Fig. 6. A diagram showing the structure of the bovine glutaminyl cyclase. The significance of the shading for this enzyme can be found at the bottom of Figure 3. The numbers of amino acids that comprise this enzyme is listed to the right.

is especially high in secretory vesicles (Fischer and Spiess, 1987). Importantly, $\text{Gln}^1\text{-GnRH}$ and $\text{Gln}^1\text{-TRH-Gly}^4$ are converted to GnRH and TRH-Gly^4 , respectively, with extracts from bovine hypophysis and rat hypothalamus. The rate of this conversion is much more rapid than that which occurs spontaneously. QC (EC 2.3.2.5) was later isolated, purified, sequenced, and cloned from bovine anterior pituitary (see Fig. 6; Pohl et al., 1991). The soluble protein has a molecular weight of approximately 38,000 and it is expressed in anterior and neurointermediate pituitary, striatum, hypothalamus, and most other brain regions. More recent evidence suggests that QC may exist in multiple molecular forms and these forms appear in a tissue-specific manner (Sykes et al., 1999).

In an experiment, we showed that QC was expressed in the murine immortalized GnRH neurons and in the rat preoptic area/hypothalamus (Wetsel et al., 1995). More recently, in our investigations with the GT1 neurons, we reported that basic fibroblast growth factor stimulated a preferential release of GnRH-Gly^{11} (Wetsel et al., 1996). An examination of the lysates from the neurons revealed that conversion of the N-terminal $\text{Gln}^1\text{-GnRH}$ to GnRH was also retarded by this factor such that release of Gln^1 peptides was increased more than 5-fold over baseline. To examine the biological activity of these GnRH intermediates, they were incubated in dispersed primary anterior pituitary cell cultures. Only $\text{Gln}^1\text{-GnRH}$ showed some activity. Despite this fact, it is not clear whether the stimulation of LH and FSH to this peptide were due to its own actions or to some possible conversion to GnRH. Nevertheless, these data show that processing of the pro-GnRH can be regulated and that some of the intermediate peptides may possess biological activity at the pituitary. Since the GnRH neurons in the hypothalamus are also subject to negative ultrashort feedback (Valença

et al., 1987), it may be the case that some of the pro-GnRH intermediates can also control secretion of the decapeptide.

Perspective

Regulation of peptide bioactivity is a complex process. Cells have devised ingenious mechanisms to control the amounts of peptides available for release. Some of these processes include gene transcription, pre-RNA splicing, protein biosynthesis, trafficking of the precursor to the regulated secretory pathway, conversion of the pro-peptide to fully processed product, secretion, and degradation. There is considerable controversy regarding the regulation of GnRH transcription in vivo and in many cases, control appears to occur posttranscriptionally (Gore and Roberts, 1997; Seong et al., 1999, 2001; Pitts et al., 2001). The topology of the hypophysiotrophic GnRH neurons reveals that a considerable distance separates the perikarya of these neurons in the preoptic area/diagonal band of Broca from the nerve terminals in the median eminence. Hence, one might anticipate that it would take a substantial amount of time for newly synthesized and processed GnRH to appear in the nerve terminal following gene expression in the perikarya. Since relatively rapid and pronounced changes can occur in the concentrations of GnRH in the arcuate nucleus and median eminence during proestrus or other steroid-induced changes (Kalra and Kalra, 1985; Culler et al., 1987) and because the median eminence contains high levels of pro-GnRH relative to that for other neuropeptides (see Wetsel et al., 1988), it is very likely that some of these rapid alterations may be due to processing of the prohormone or its intermediates to GnRH in the nerve terminals. In this way, bioactive GnRH could be supplied relatively quickly in preparation for secretion. Since many different hormones, transmitters,

peptides and lipids can affect GnRH secretion (Wetzel, 1995), it will be important to determine whether these agents can also influence the conversion of the pro-GnRH to the bioactive decapeptide. In this context, recent data on the possible roles of the PCs, CPE, PAM, and QC in pro-GnRH processing afford new perspectives on studies of GnRH function.

It has long been known that the activity of GnRH is primarily curtailed through degradation (Koch et al., 1974; O'Cuinn et al., 1990; Lew et al., 1994). Recently we have found that the immortalized GnRH neurons can degrade the decapeptide into a variety of different fragments. The appearance and patterns of fragments are consistent with our mRNA results where we find that angiotensin converting enzyme, neutral endopeptidase, metalloendopeptidase, and prolyl endopeptidase are all expressed in these neurons. Collectively, these data show not only that biosynthesis and processing of the prohormone occur within GnRH neurons, but that degradation can also occur. Inasmuch as some the GnRH fragments have biological activities on their own (Chen et al., 1993; Bourguignon et al., 1994), these findings suggest that GnRH, itself, may serve as a pro-peptide and that the pro-GnRH-derived peptides may elicit pleiotropic responses.

Abbreviations

CPD	carboxypeptidase D
CPE	carboxypeptidase E
FSH	follicle-stimulating hormone
GAP	gonadotropin-releasing hormone associated peptide
GnRH	gonadotropin-releasing hormone
[Hyp ⁹]GnRH	[hydroxyproline ⁹]GnRH
LH	luteinizing hormone
PACE	paired amino acid converting enzyme
PAL	peptidyl- α -hydroxyglycine α -amidating lyase
PAM	peptidylglycine α -amidating mono-oxygenase
PC	prohormone convertase
pGlu ¹	pyroglutamate
PHM	peptidylglycine α -hydroxylating monooxygenase
QC	glutaminy cyclase
WT	wild type

Acknowledgments

We would like to thank all of the past students, post-doctoral fellows, and collaborators who contributed to these studies. Some of the work described in this paper was supported by NIH grant HD36015.

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The GnRH neuron: molecular aspects of migration, gene expression and regulation

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Introduction

The release of gonadotropin-releasing hormone (GnRH) from the GnRH neurons of the basal hypothalamus directs the synthesis and secretion of the pituitary reproductive hormones, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary. As the ultimate neural regulators of the hypothalamic–pituitary–gonadal axis, GnRH neurons integrate a variety of environmental, psychological and homeostatic cues into a signal that effects reproductive function.

GnRH neuronal migration and anatomy

Unlike other hypothalamic neuropeptide producing cells, GnRH neurons do not arise from the developing basal forebrain, they originate in the olfactory placode in mammals and migrate across the nasal cavity and cribriform plate into the forebrain (Schwanzel-Fukuda et al., 1987; Wray et al., 1989). In mice, for example, the GnRH neurons are derived from the neural ectoderm and are born on day 10.5 pc, and begin to express GnRH mRNA and protein at between day 10.75 and 11.5 pc. GnRH neurons

then migrate across the olfactory cavity to the forebrain between day 12.5 pc and day 16.5 pc (Wray et al., 1989; Wu et al., 1997). GnRH neurons migrating across the nasal cavity appear to be guided, in part, by the polysialic acid-rich form of the neural cell adhesion molecule (PSA-NCAM) (Yoshida et al., 1999) in association with the vomeronasal nerves (VNN). Other investigators have posited that factors such as GABA (Fueshko et al., 1998), adhesion related kinase (Fang et al., 1998) or peripherin (Wray et al., 1994) might play a role in the guidance of the GnRH neurons. The initial migration to the forebrain vesicles follows the vomeronasal and terminal nerves, however, after entering the rostral forebrain, GnRH neurons become disassociated from the caudal branch of the VNN. At this time it is thought that GnRH neurons are no longer guided by an, as yet, defined anatomical structure (He et al., 1989). Instead, neuronal migration may trail behind as axonal migration, directed by chemoattractive signals from the medial basal hypothalamus (Rogers et al., 1997) is occurring.

The lack of migration of GnRH neurons into the forebrain can result in reproductive dysfunction. For example, some cases of hypogonadotropic hypogonadism are due to a deletion of an X-linked gene referred to as anosmin-1 or Kalig (Schwanzel-Fukuda et al., 1989; Franco et al., 1991; Wierman et al., 1992). This disorder, Kallmann's Syndrome, results in a lack of proper neuronal migration in humans. The Kalig locus contains four fibronectin type III repeats (Legouis et al., 1991, 1994), a motif

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associated with adhesion molecules (Yokosaki et al., 1998). Forms of idiopathic hypogonadotropic hypogonadism not associated with Kallmann's syndrome have been described in humans, but the mechanisms of dysregulation of the GnRH neurons remain unknown (Waldstreicher et al., 1996; Quinton et al., 1997). Certainly, a lack of developmental progression of GnRH neurons due to the absence of proteins important for migration may result in some of these cases of idiopathic hypogonadotropic hypogonadism.

A mouse model exists for a non-Kallmann's-like associated hypogonadotropic hypogonadism. The hpg mouse was identified as reproductively dysfunctional, and contained a large genetic deletion that included much of the GnRH gene (Mason et al., 1986). Neurons expressing the truncated form of the GnRH gene are still present in the usual locations in the forebrain indicating that migration of the neurons is unaffected by the absence of the GnRH decapeptide in these animals (Livne et al., 1993b). Transplantation of hypothalamic grafts (Rogers et al., 1998), or GnRH expressing cell lines (Kokoris et al., 1988; Silverman et al., 1992) into the hypothalami of the hpg mice rescued the phenotypic hypogonadism. This further underscores the central importance of the GnRH neurons as the primary regulators of pituitary gonadotropin secretion. Surprisingly, despite the efforts of a number of investigators, no mutation of the GnRH gene has been identified to date in humans.

It should be stressed that it is unclear whether GnRH neurons are merely passively responding to signals in their immediate milieu, or whether local signals trigger changes in gene expression in GnRH neurons that are ultimately responsible for migration, and for changes in cellular morphology or GnRH gene expression. There is evidence for the latter model in another neuronal system; the developmental organization of the cerebellum. Normally granule cells migrate along radial glia to their final location in the cerebellum. In the mutant *weaver* mouse the granule cells fail to migrate to their final location in the cerebellum (Rakic and Sidman, 1973a,b). Studies in chimeric mice suggested that the defect in the *weaver* mouse was intrinsic to the migrating granule cell (Goldowitz and Mullen, 1982; Goldowitz, 1989). Evidence for these types

of changes during development in GnRH neurons comes from studies looking at differences in gene expression in GN10 versus GT1 GnRH expressing cells (Fang et al., 1998; Allen et al., 1999). The first is derived from an olfactory tumor (migratory GnRH neurons), and the latter from an hypothalamic tumor (post-migratory GnRH neurons). These studies identified more than 10 factors differentially expressed in the two cell lines including an adhesion related kinase, Ark, produced in GN10 cells but not in GT1 cells that may have an anti-apoptotic function (Fang et al., 1998; Allen et al., 1999). Recent studies from Kramer and Wray have used single cell PCR of identified migratory and post-migratory GnRH neurons from hypothalamic explants to identify a factor called nasal embryonic LHRH factor (NELF) that appears to play a role in olfactory neuronal migration (Kramer and Wray, 2000). Additional evidence comes from a biochemical analysis of GnRH neurons during development showing that the growth and plasticity associated peptide, GAP43, was expressed at much higher levels in neurons within the nasal septum than in neurons that have already migrated into the forebrain (Livne et al., 1993a). Taken together, these studies indicate that there are additional differentiation events that occur following the onset of GnRH gene expression at day 10.75 pc.

In the adult mouse, the vast majority of GnRH containing neurons are located in the hypothalamus at the level of the organum vasculosum of the lamina terminalis (OVLT) and the pre-optic area (POA), but some GnRH neurons have also been localized in the cerebral cortex and the limbic system (King et al., 1982; Barry et al., 1985; Schwanzel-Fukuda et al., 1987). In addition, GnRH neurons are scattered within the olfactory bulbs. Therefore, it appears as if GnRH neurons are strewn along the migratory pathway to the basal hypothalamus. It is not clear what signals are responsible for the organization of the microarchitecture of the mammalian GnRH neurons in the basal forebrain, although there is some evidence that there are important functional groupings within the scattered GnRH neurons. For example there are quiescent subpopulations of GnRH neurons activated during various physiological events, such as during the preovulatory surge in female mammals (Hiatt et al., 1992; Porkka-Heiskanen et al., 1994), that have a specific anatomical arrangement.

Subpopulations of GnRH neurons

In the hypothalamus, during a variety of physiologic stimuli, GnRH neurons have been shown to be 'recruited' into increasing their expression of GnRH (Dutlow et al., 1992; Hiatt et al., 1992; Porkka-Heiskanen et al., 1994; Gore et al., 1999; Gore, 1998). For example, during the preovulatory GnRH surge in rats, it appears that a normally quiescent population of GnRH neurons is activated (Hiatt et al., 1992; Porkka-Heiskanen et al., 1994; Rajendren, 2001). Although these differences in GnRH neuronal activation may be a result of variations in the afferent inputs to each population of GnRH neurons, the pattern of release of GnRH from the median eminence during the GnRH surge is radically different from that seen during non-surge periods (Levine and Ramirez, 1982; Moenter et al., 1991), and may reflect profound differences in the physiology of the surge generating, and the non-surge generating populations of GnRH neurons. The intracellular events required for activation of GnRH neurons during the surge are unknown.

Another instance where GnRH neurons are recruited to produce higher levels of GnRH gene expression is during the period preceding the onset of puberty in the mouse (Gore et al., 1999; Wolfe et al., 1995), and rat (Gore, 1998), perhaps to prepare the neurons for the increased levels of GnRH secretion that occurs during puberty. Increases in GnRH gene expression after the onset of puberty when compared with prepubertal animals have been observed in the hamster (Parfitt et al., 1999). Again, it is not clear what factors (neurotransmitters, growth factors, second messengers or transcription factors) are involved in these puberty-related changes in GnRH

gene expression although there is some evidence that the amino acid family of neurotransmitters [GABA (Mitsushima et al., 1994; Terasawa et al., 1999) or NMDA (Gore et al., 1996; Zamorano et al., 1998; Terasawa et al., 1999)] may play a role in regulating the initiation of puberty.

Skykker et al. have indicated that, indeed, there might be independently derived populations of GnRH neurons located in the brain (Skykker et al., 1999). That is, GnRH neurons derived from regions other than the olfactory placode are present in the mouse brain during early development and persist until the third week of postnatal life. It is not clear whether these ectopically expressed GnRH neurons subserve a functional role in the mouse.

GnRH expressing neuronal cell lines

As a means of developing an in vitro model for the study of GnRH neuronal cell activity, immortalized GnRH-expressing neuronal cell lines were created by targeted tumorigenesis (Mellon et al., 1990; Radovick et al., 1991). Our laboratory targeted the expression of the simian virus 40 T antigen (SV40-Tag) to the GnRH neurons with 1131 bps of the human GnRH gene 5'-upstream regulatory sequence. One of these animals developed an olfactory tumor (Radovick et al., 1991) from which several GnRH immunoreactive cell lines (NLT, GN10, GN11) were subsequently derived. Characterization of the NLT and GN11 cells demonstrated that these cells display neuronal morphology and neuron specific markers [Table 1; (Fischer et al., 1991; Mavilia et al., 1993)]. Solution hybridization-RNase protection assays indicate that NLT cells express proGnRH mRNA at higher levels than GN11 cells. Radioimmunoassays

TABLE 1
Comparison of GN11 and NLT cells

Cell line	Tag expression	Neuronal markers	Relative GnRH mRNA levels	Predominant GnRH mRNA species	GnRH secretion by RIA
Gn11	+	+	0.03	Splice variant	1.5 pg/ml
NLT	+	+	1	Full length	20 pg/ml

Both GN11 and NLT cells express neuronal markers such as Map-2 and Tau, and express the T antigen oncogene. The predominant mRNA species in the GN11 cells is a splice variant in which the second exon is excised. NLT cells primarily express the full length variant. NLT cells release more GnRH peptide than GN11 cells most likely due to the higher relative levels of the full length GnRH mRNA species seen in NLT cells

reveal that both cell lines are able to synthesize and secrete GnRH. However, NLT cells secrete about 10 times higher levels of GnRH than GN11 cells [Table 1; (Zhen et al., 1997a)]. We have identified several factors that are differentially expressed in these two cell lines that may contribute to their differences in phenotype (Zhen et al., 1997a). Our laboratory has shown that the POU homeodomain transcription factor Brn-2 is present in NLT cells, but not in GN11 cells [Fig. 1; (Wolfe et al., 2002)]. Transfection of GN11 cells with a Brn-2 expression vector stimulates mouse (Fig. 2) and human (Fig. 3) GnRH gene promoter activity. In addition, our laboratory has identified a novel factor, NLT expression factor-1 (NLT-EF1) that is more highly expressed in NLT cells than GN11 cells (Fig. 4). Interestingly, NLT-EF1 shares homology with a mucin-type glycoprotein that stimulates motility of keratinocytes during wound regeneration (Scholl et al., 1999). We are presently exploring whether NLT-EF1 regulates motility of GnRH neurons.

The GT1 cell lines developed by Pamela Mellon's laboratory were harvested from an hypothalamic tumor and may represent a more mature GnRH neuronal cell line (Mellon et al., 1990). Indeed, one group has used a polymerase chain reaction (PCR) based differential display system to identify genes expressed differentially in olfactory bulb derived GN11 cells and hypothalamus derived GT1 cells (Fang et al., 1998; Allen et al., 1999). These genes have been hypothesized to produce proteins important for the regulation of migration and for preventing apoptosis. The three GT1 cell lines that have been primarily used are the GT1-1, GT1-3 and GT1-7 cell lines (Mellon et al., 1990). These cell lines, like the cell lines obtained in our laboratory from the olfactory tumor, possess significant physiological, electrophysiological, morphological, and molecular differences with each other (Mellon et al., 1990; Wetsel et al., 1991; Martinez et al., 1992; Bosma, 1993; Weiner and Martinez, 1993; Poletti et al., 1994).

The GnRH gene

The GnRH neurons are particularly difficult to study due both to their low abundance in the brain [about 800 neurons in the mouse (Wray et al., 1989)], and

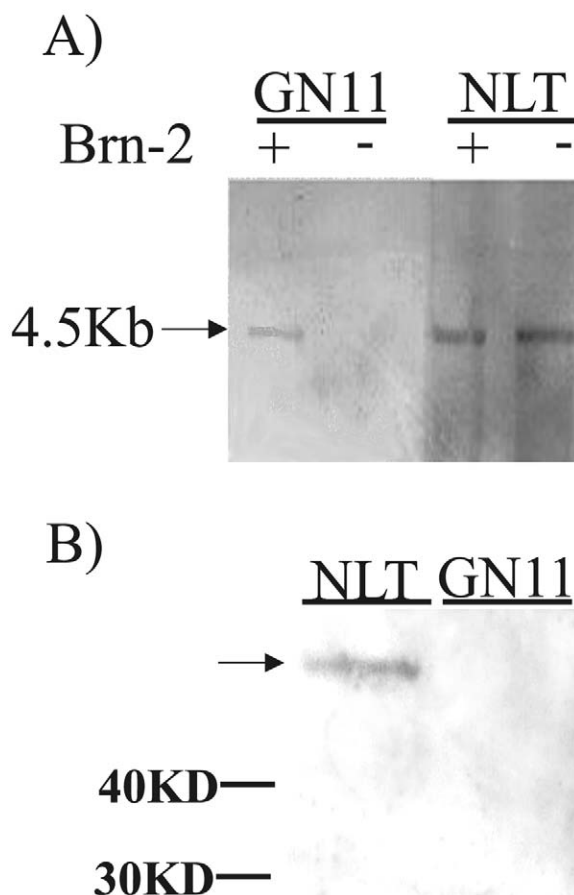


Fig. 1. Brn-2 mRNA and protein are found in GnRH neurons. (A) Northern blot analysis of mRNA obtained from the GN11 or NLT cell lines transfected with either a Brn-2 expression vector (+) or a control vector (-). A digoxigenin labeled riboprobe was found to hybridize at a size of 4.5 kb to mRNA of both GN11 and NLT cells transfected with Brn-2pSG5, and to NLT cells transfected with pSG5, but not to GN11 cells transfected with pSG5. (B) Western blot of nuclear extract proteins from NLT and GN11 cells. Size markers are indicated on the left. A band is observed in the NLT lane (arrow) that runs at about 54 kDa. No band was observed in the GN11 lane. (C) Coronal, whole mount tissue section of a mouse brain at the level of the OVLT. A black precipitate indicates Brn-2 mRNA, and GnRH peptide is labeled with a Cy-3 phosphoramidite (fluorescing). Brn-2 mRNA is observed in the cell body of the GnRH neuron (arrow).

to their diffuse and widely scattered distribution. Despite these obstacles the GnRH gene has been cloned in a number of species (Bond et al., 1989; Hayflick et al., 1989; Radovick et al., 1990; Kepa et al., 1992), and has been found to be about 4 kb

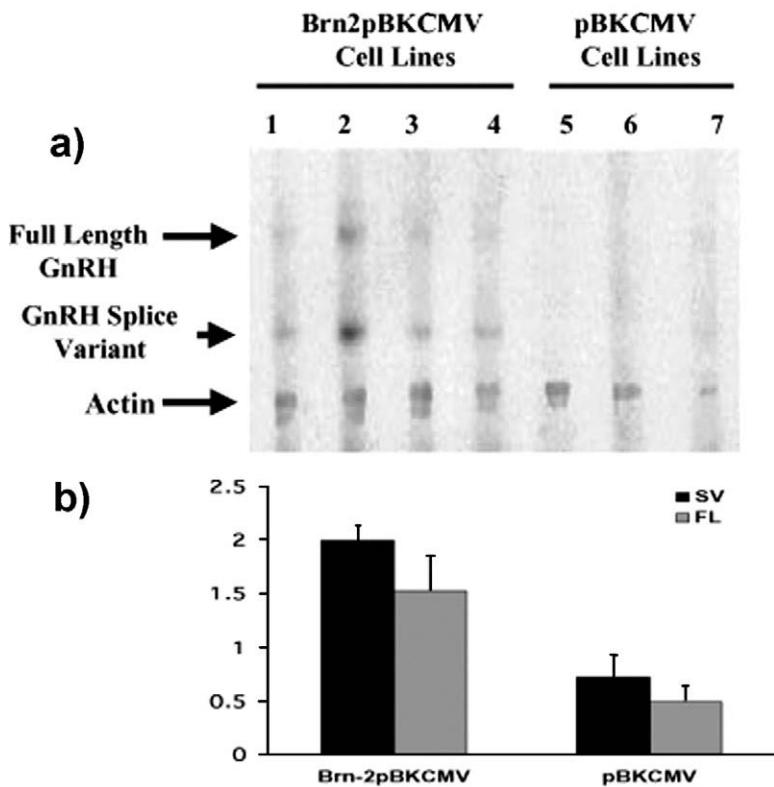


Fig. 2. Brn-2 Stimulates mGnRH Gene Expression. (A) RNase protection assay that was performed on mRNA obtained from stable transfection of GN11 cell lines with either the empty pBKCMV expression vector (lanes 5–7) or the Brn-2pBKCMV expression vector (lanes 1–4). GnRH full length mRNA, the truncated splice variant and actin mRNA are indicated by arrows to the left. Two separate RPAs were performed on the mRNA and the values of both the full length GnRH transcript (FL: Solid bars) and the splice variant (SV: shaded bar) averaged and shown in (B) where values are reported as arbitrary units of GnRH corrected for arbitrary units of actin. In (A) the GnRH bands are scanned from a film after 4 days exposure at -70°C . All quantification of band intensity was performed with the phosphorimager. Background levels for each band were subtracted from band intensity. Images of actin bands are from phosphorimager to reduce intensity for better visualization, and are superimposed on scanned image of film.

in length and to contain four exons. The first exon consists of the 5' untranslated region, the second exon encodes the signal sequence of GnRH, the GnRH decapeptide, and the first 11 amino acids of GnRH-associated peptide (GAP), and the last two exons encode the remaining amino acids of GAP as well as the 3' untranslated region. Low levels of GnRH immunoreactivity have also been localized in the placenta, the gonads and the mammary glands (Seeburg et al., 1987). Interestingly, placental GnRH transcription is initiated from a different promoter initiation site than that used for transcription of hypothalamic GnRH mRNA (Dong et al., 1993). In addition, the hypothalamic and placental GnRH genes undergo differential RNA splicing, with the

first intron being retained in the placental mRNA and removed in the hypothalamic mRNA (Radovick et al., 1990; Dong et al., 1993). It is unclear what the significance is of the extrahypothalamic expression of GnRH. In the mouse hypothalamus, studies have shown that two differential splicing events can occur. One form of mRNA contains all four exons while the other does not contain the second exon, which encodes the GnRH decapeptide (Zhen et al., 1997a). The factors responsible for this differential splicing are unknown, but clearly a better understanding of the transcription factors that regulate these different splicing events will advance our understanding of both the cell-specific expression and the regulated expression of the hGnRH gene.

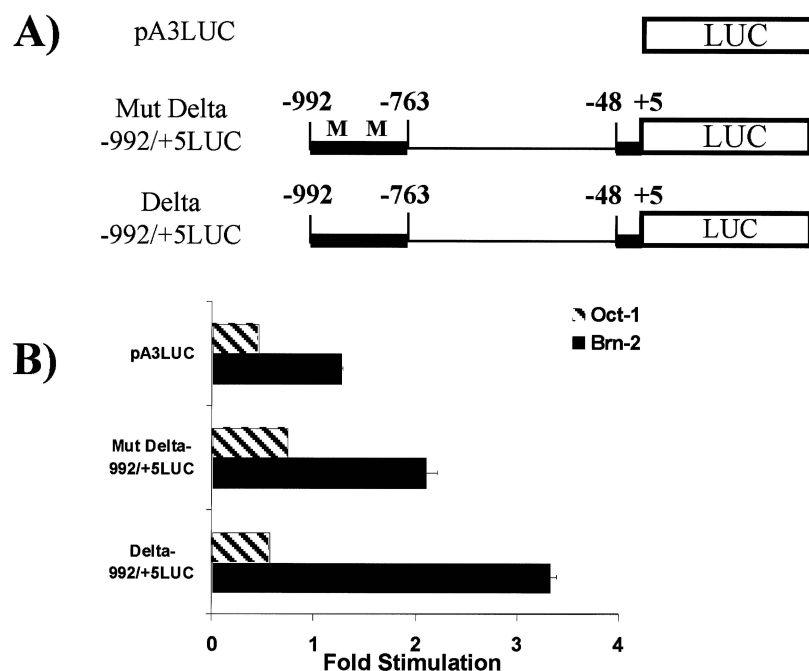


Fig. 3. Brn-2 Stimulates hGnRH Gene Expression. (A) Schematics of constructs used in transient transfection experiments. pA3LUC, Δ -992/+5LUC (labeled Delta-992/+5LUC) and Mut Delta-992/+5LUC. M indicates mutated Brn-2 consensus binding sites in the TSE. (B) Transient transfections of GN11 cells using Lipofectamine Plus reagent (Gibco, BRL). Cells were transfected with reporter vector (Δ -992/+5LUC [labeled Delta-992/+5LUC], Mut Delta-992/+5LUC [the same as Δ -992/+5LUC except with mutations of both Brn-2 consensus sites], and the empty pA3LUC) and 0.5 μ g of empty pSG5 expression vector, Brn-2pSG5 or Oct-1pSG5. Data are reported as fold stimulation by Brn-2 or Oct-1 as compared to pSG5 + SE.

Cell-specific expression of the GnRH gene

Several *in vitro* studies have used mouse-derived immortalized GnRH-secreting neuronal cell lines to investigate the neuronal expression of the rat and mouse GnRH gene. Transient transfection studies using the rat GnRH (rGnRH) gene promoter identified a 173 bp proximal promoter region (Eraly and Mellon, 1995) and a 300 bp region located 1.8 kb upstream from the transcription start site (Whyte et al., 1995) that conferred cell-specific expression. Both these sites were reported to be important for the appropriate neuronal expression of the rGnRH gene *in vitro* (Nelson et al., 2000).

In vivo, GnRH neurons are dispersed and are influenced by growth factors, steroids, and neurotransmitters secreted by various adjacent cell types. Because *in vitro* studies are unlikely to reflect the elaborate intricacy of *in vivo* GnRH gene regulation, *in vivo* models have been developed to study the

regulation of the GnRH gene. A transgenic mouse study, using a *lacZ* reporter, identified the critical elements for expression of the mGnRH gene between -2.1 kb and -1.7 kb of the distal 5' sequence (Pape et al., 1999).

As a model to study the cell-specific expression of the GnRH gene, our laboratory has used a transgenic mouse model containing gene constructs consisting of promoter deletion fragments of the GnRH gene fused to the luciferase reporter gene (LUC). To identify promoter elements of the human GnRH (hGnRH) gene that were essential for cell-specific expression the following promoter deletion fragments were used; -3828/+5, -1131/+5, -992/+5, -795/+5, and -484/+5 (Reporter genes named 3828LUC, 1131LUC, 992LUC, 795LUC and 484LUC, respectively). Tissue panels were performed on each of these mouse lines to ascertain whether reporter gene expression (LUC) was confined to GnRH neuron containing re-

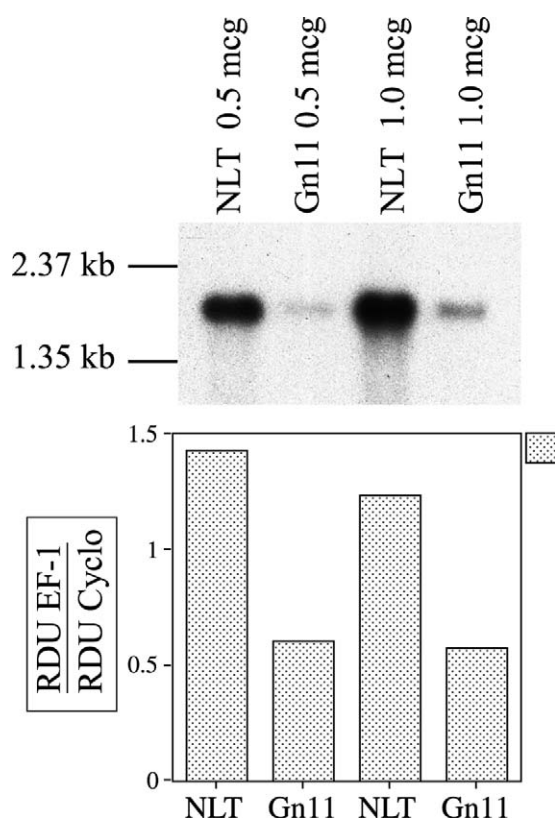


Fig. 4. NLT-EF1 mRNA is More Abundant in NLT Cells Than GN11 cells. Northern blot analysis of mRNA from GN11 and NLT cells using a ^{32}P -labeled cDNA probe for NLT-EF1. 0.5 μg or 1 μg of mRNA was used as indicated. Size marker is indicated on the left. Quantitative representation of band intensity is shown in the graph. Values are given as band intensity of the NLT-EF1 band divided by the band intensity of a cyclophilin standard. NLT-EF1 mRNA levels were found to be between 2–3 fold higher in NLT cells than in GN11 cells.

gions. When promoter fragments larger than 992 bps were used [Fig. 5 for 992LUC mice, data from 3828LUC and 1131LUC mice in (Wolfe et al., 1995)] relatively high levels of luciferase enzyme activity, as measured in a luminometer, were observed in the hypothalamus and olfactory bulbs. Low to undetectable levels of luciferase activity were measured in tissue homogenates taken from other central and peripheral tissues. Double labeling immunohistochemistry was performed to confirm colocalization of the GnRH peptide and the luciferase protein in the hypothalamus (Fig. 6). These studies indicate that promoter elements essential for the cell-specific

expression of the hGnRH gene are located within the first 992 bps of the promoter.

When mice containing either the 795LUC (Fig. 7) or 484LUC transgene were examined, no luciferase activity was observed in any of the tissue homogenates. These studies demonstrate that promoter elements sufficient to target gene expression to GnRH neurons are not located within the first 795 bps of the hGnRH gene promoter. These results indicate that promoter elements of the hGnRH gene between –992 and –795 are essential for cell-specific expression of the gene.

To determine whether this promoter region contained elements that were sufficient for cell-specific expression an additional transgenic animal line was created. These mice contained a reporter gene with a promoter fragment from –992 to –763 fused to a minimal 48 bp hGnRH promoter fragment (Fig. 8a). When tissue panels from these mice were examined, relatively high levels of luciferase expression were observed in the hypothalamus and olfactory bulbs but not in other tissues. In whole, these studies demonstrate that the –992/–795 hGnRH promoter fragment is both essential and sufficient to direct gene expression to GnRH neurons.

Interestingly, the human and mouse cell-specific regions bear little homology to each other and neither share homology with the critical rat promoter regions identified in the *in vitro* studies. To reconcile these discrepant observations, a similar model system was used to identify the promoter elements of the mGnRH gene that are important for cell-specific expression (Kim et al., 2002). We constructed transgenic mice with various fragments of the mouse GnRH gene promoter fused to the luciferase reporter gene as a marker for *in vivo* mGnRH gene promoter activity. All three 5' fragments of the mGnRH promoter that we studied, –3446/+23 bp, –2078/+23 bp, and –1005/+28 bp, targeted expression of the luciferase transgene to the hypothalamus (Fig. 9). Distal to the proximal –173 bp conserved region, there is approximately 50% overall homology between the proximal –1005 bp of the human and mouse GnRH promoters. As discussed above, studies of the hGnRH gene in transgenic mice localized a hypothalamic-specific element between –992 bp and –795 bp of the hGnRH gene. Within the human hypothalamic-specific element, there are several re-

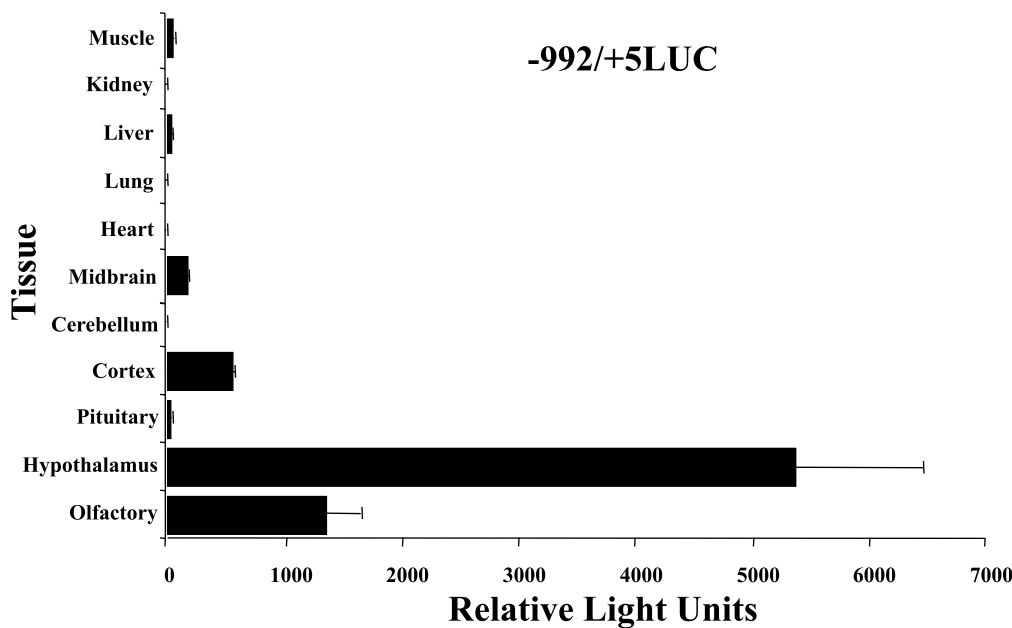


Fig. 5. Tissue Panel from 992LUC Mice. Relative luciferase activity (Shown as relative light units, RLU) of tissues taken from transgenic mice containing the $-992/+5$ LUC transgene. The luciferase reporter is regulated by the $-992/+5$ hGnRH promoter fragment. All tissue levels are the mean \pm SE of four adult animals from transgenic line 26 except the hypothalamic values, which are the means \pm SE of 6 mice. (B) Relative luciferase activity (RLU) of tissues taken from transgenic mice containing the $-992/+5$ LUC transgene. All tissue levels are the mean \pm SE of three adult animals from transgenic line 64 except the hypothalamic and olfactory values, which are the means \pm SE of 16 mice. (C) Averaged data from three tissue panels used in Figure 1B where the values are corrected for protein levels by dividing the RLU by mg protein.

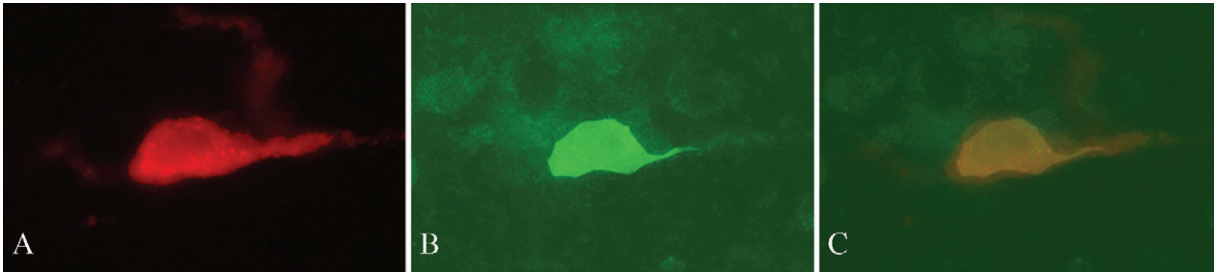


Fig. 6. Luciferase is colocalized with GnRH in 992LUC mice. Double fluorescence histochemistry of mouse brain sections from a 992LUC mouse. Shown is a neuron containing GnRH peptide (shown as red in (A), luciferase protein (shown as green in B). (C) is an overlay of A and B showing colocalization of the two proteins. Cy3 (red) and Alexa Fluoro 488 (green) were visualized using the appropriate filters.

gions that share greater homology with the mGnRH gene, and further study of these homologous regions may elucidate the critical factors that target hypothalamic GnRH expression.

Our findings using the luciferase reporter and the mouse GnRH promoter differ from a recent transgenic mouse study in which the mGnRH promoter fused to a lacZ reporter localized the critical ele-

ments for expression of the mGnRH gene between -2.1 kb and -1.7 kb of the promoter (Pape et al., 1999). In these mice, deletion of mGnRH promoter sequences $5'$ to 1.7 kb resulted in a complete absence of detectable β -galactosidase expression within the brain, whereas we detected luciferase expression in transgenic mice bearing the -1005 mGnRH-LUC transgene. In the adult, the proximal -1005 bp of

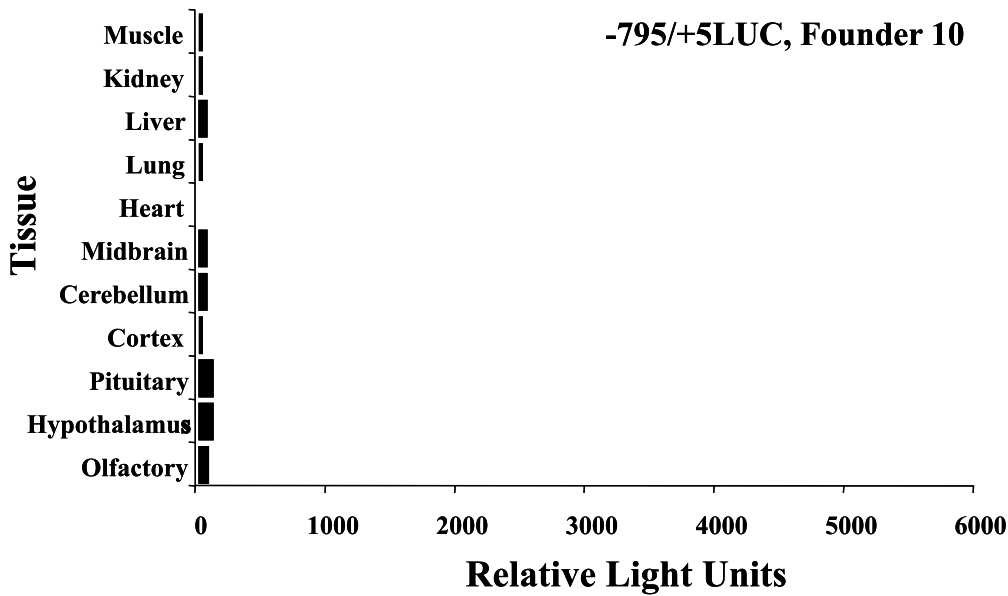


Fig. 7. Tissue Panel from a 795LUC mouse. Relative luciferase activity of tissues taken from a representative transgenic mouse containing the $-795/+5$ LUC transgene (one of 8 founders examined). The luciferase reporter is regulated by the $-795/+5$ hGnRH promoter fragment.

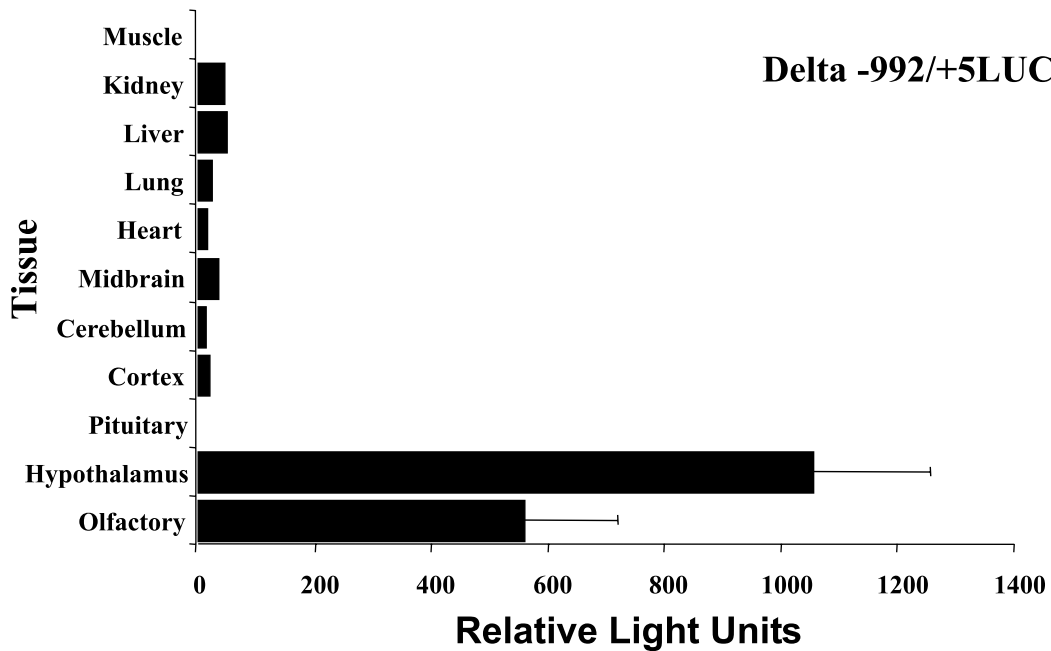


Fig. 8. Tissue Panel from a $\Delta-992/+5$ LUC Mouse. Relative luciferase activity of tissues taken from a representative transgenic mouse containing the $\Delta-992/+5$ LUC transgene (one of 3 positive founders examined). The luciferase reporter is regulated by the $-992/-763/-48/+5$ hGnRH promoter fragments. The hypothalamic and olfactory values are the average of animals from all three founders \pm SE.

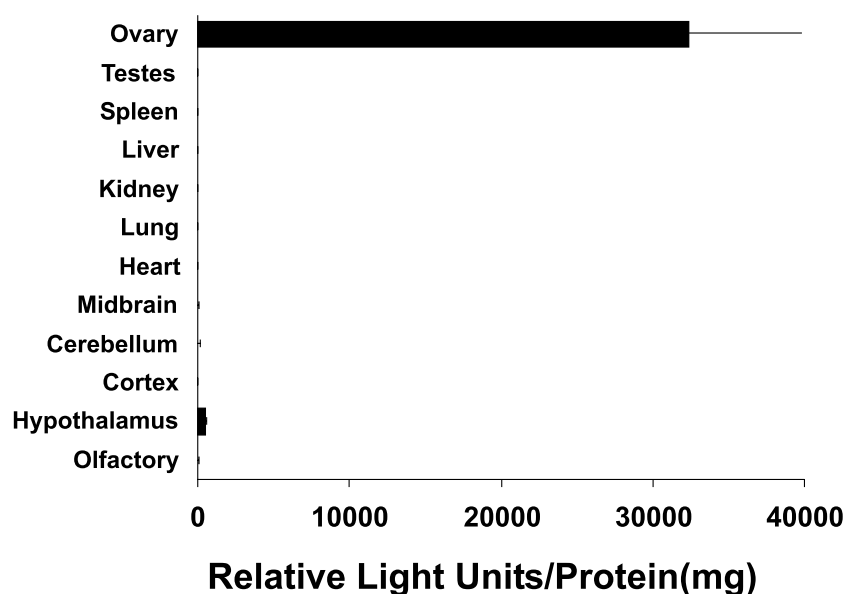


Fig. 9. Tissue Panel from a 1005LUC Mouse. Relative luciferase activity of tissues taken from a representative transgenic mouse containing the $-1005/+5$ LUC transgene (one of 3 positive founders examined). The luciferase reporter is regulated by the $-1005/+23$ mGnRH promoter fragment. The hypothalamic and olfactory values are the average of animals from all three founders \pm SE.

the mGnRH targeted luciferase to the hypothalamus in the -1005 mGnRH-LUC mice, but expression levels were lower (474 ± 86 RLU) compared with the levels seen in the -2078 mGnRH-LUC mice ($1,688 \pm 444$ RLU). Perhaps, the mGnRH promoter region between -2.1 kb and -1.7 kb, although not critical for targeting hypothalamic expression, contains sequences essential for enhancing hypothalamic expression of GnRH.

The mGnRH promoter fragments containing -2078 bp and -1005 bp of 5' sequence targeted luciferase transgene expression to the hypothalamus, but at lower levels than the transgenic mice generated with -3446 bp of mGnRH promoter. In general, comparison of transgene expression levels between different founder lines must be done cautiously since transgene expression levels are affected by the chromosomal integration site and transgene copy number (al Shawi et al., 1990). Nevertheless, transgenic mice derived from 8 different embryos bearing the -2078 mGnRH-LUC transgene were examined. It is striking that even in the transgenic line that expressed luciferase at the highest level, luciferase activity in the hypothalamus was approximately 5 fold lower than was seen in the transgenic mice bearing the -3446 mGnRH-LUC transgene. These data would

suggest that an enhancer for the *in vivo* expression of hypothalamic mGnRH is contained in the mGnRH promoter region between -3446 bp and -2078 bp.

Our *in vivo* observations corroborate the *in vitro* studies performed in the GT1-7 cell line. The rat neuron-specific GnRH enhancer, located between -1863 and -1571 bp, shares 90% homology to the region of the mouse GnRH promoter located between -2384 bp and -2081 bp. Although the region between -2384 bp and -2081 bp was not found to be essential for targeting mGnRH expression to the hypothalamus in our mGnRH-LUC mice, deletion of sequences 5' to -2078 bp resulted in a dramatic decrease in hypothalamic luciferase expression levels. These results would support the hypothesis that critical enhancer sequences for the *in vivo* expression of hypothalamic mGnRH are located between -2384 bp and -2081 bp of the mGnRH promoter.

In vitro studies with the rGnRH promoter in GT1-7 cells have also identified several transcription factors that interact with these promoter regions to regulate rGnRH expression: C/EBP (Belsham and Mellon, 2000), GATA (Lawson et al., 1996 A.D.; Lawson and Mellon, 1998 A.D.), Oct-1 (Clark and Mellon, 1995; Eraly et al., 1998), Otx 2 (Kelley et al., 2000), SCIP/Oct-6/Tst-1 (Wierman et al., 1997).

The rat and the mouse proximal promoter regions are highly conserved between the rat and mouse, and *in vitro* studies with the mouse GnRH (mGnRH) gene promoter suggest that Oct-1 may also regulate the neuronal expression of the mGnRH gene (Chandran et al., 1999). Analysis of the corresponding mouse gene sequences, between -2384 bp and -2081 bp, however, reveal differences in the presumed transcription factor recognition sites. There is some unpublished evidence that these changes may eliminate binding to the mGnRH promoter, *in vitro* (Chandran and DeFranco, 1999). Further study is needed to determine whether Oct-1 also has a role in enhancing the *in vivo* expression of mGnRH and whether any of the above transcription factors, or an as yet undescribed transcription factor(s), are involved.

Non-neuronal expression of GnRH

More recently, an extra-pituitary role for GnRH has been appreciated. Low levels of GnRH expression have been found in peripheral reproductive tissues, such as placenta (Radovick et al., 1990), breast, ovary and testes (Dong et al., 1993). There is increasing evidence that ovarian GnRH directly modulates ovarian function in a paracrine or autocrine manner. GnRH has been demonstrated to have both stimulatory and inhibitory influences on ovarian cell differentiation, steroidogenesis, and oocyte maturation (Hsueh and Erickson, 1979a,b; Knecht et al., 1985; Richards, 1994).

Interestingly, when the mGnRH promoter region distal to -2078 bp was deleted in our mice bearing the mouse GnRH-luciferase transgene, high levels of ovarian luciferase were detected, and even higher levels of ovarian luciferase were detected in mice bearing the -1005 mGnRH-LUC transgene. Our studies demonstrate that sequences contained within the proximal -1005 bp of the mGnRH promoter are sufficient to target ovarian, as well as hypothalamic, expression of mGnRH. Our data also suggest that an ovarian GnRH repressor element may be located in the distal region of the mGnRH promoter between -3446 bp and -2078 bp since deletion of this region unmasks luciferase expression in the ovaries of transgenic mice bearing the mGnRH-LUC transgene. We speculate that repressor proteins in the ovary normally interact with this ovarian GnRH

repressor element and permit GnRH expression only in certain physiologic situations.

Our finding of high levels of ovarian luciferase expression was initially surprising since no ovarian luciferase activity was detected in our previous studies using the hGnRH promoter to direct luciferase expression (Wolfe et al., 1995, 2002). This discrepancy may be due to species-specific differences in the role of GnRH in follicular development or in the ovarian proteins that may interact to regulate ovarian GnRH expression. Alternatively, it is possible that an ovarian GnRH repressor element exists in the human GnRH promoter as well, but has not yet been identified. Further studies may reveal the identity of the specific repressor proteins and the specific physiologic states in which ovarian GnRH expression is increased.

Historically, the study of the molecular elements important for the development and regulation of the GnRH neuron has been challenging. Generally, investigators have relied on anatomical methods or direct or indirect measurement of GnRH release from the hypothalamus either *in vivo* or *in vitro*. The development of homogeneous GnRH neuronal cell lines has allowed for the direct examination of the neurons in previously impossible ways. Electrophysiology (Bosma, 1993; LeBeau et al., 2000; Van Goor et al., 2000), signal transduction (Bruder et al., 1992; Zhen et al., 1997b; Poletti et al., 2001), gene expression (Kepa et al., 1996; Yeo et al., 1996; Lawson et al., 1998; Nelson et al., 2000) and protein translation (Gore et al., 1995; Sosnowski et al., 2000) studies have all been performed using cell lines. Transgenic animal models have provided a new and valuable tool for the study of the GnRH neuron. The direct, *in vivo*, analysis of cell-specific expression (Wolfe et al., 1995; Pape et al., 1999; Suter et al., 2000; Lawson et al., 2002; Wolfe et al., 2002) of the GnRH gene and regulation of the GnRH neuron (Wolfe et al., 1995; Lawson et al., 2002) have been performed. Present technologies exist to produce GnRH neuron-specific deletions of genes and even to control the timing of expression. These models will provide a wealth of information about factors important for GnRH neuronal migration and development. They also will help determine what pathways are used to transmit information about homeostatic, psychological and environmental status to the GnRH neuron.

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Physiology and release activity of GnRH neurons

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Introduction

The gonadotropin-releasing hormone (GnRH), which was first isolated from the mammalian hypothalamus, was originally identified as a hypophysiotrophic decapeptide hormone that facilitates gonadotropin release from the pituitary gonadotropes. Recent immunocytochemical and in situ hybridization techniques have greatly advanced our knowledge of the anatomical features of the GnRH neuronal systems (Oka, 1997 for review). These anatomical studies have shown that the GnRH neurons not only synthesize and transport releasable peptides to the median eminence (hypothalamic system) but also project widely in various brain regions (extrahypothalamic system). On the other hand, combined HPLC chromatography and peptide biochemistry have revealed a wide variety of molecular species of GnRH peptides among different vertebrate species. Furthermore, it has also been recognized quite recently that there are also diversity of GnRH receptors, and the functional and evolutionary significance of such diversity of GnRH ligand and receptors has been attracting more and more attention. Although the function of the hypothalamic GnRH system has been well studied and is exactly what the name implies, i.e., facilitation of the release of gonadotropins, the functional significance of the extrahypothalamic

GnRH systems is elusive and has only poorly been studied yet.

We have been interested in the biological significance of an enigmatic extrahypothalamic GnRH system, the terminal nerve (TN)–GnRH system, of vertebrate brains. Although we still do not have a complete picture of the functional significance of TN–GnRH system, we now have several lines of evidence to suggest that TN–GnRH system serves as a neuromodulatory system that controls the motivational or arousal state of the animal. For the biological analysis of this system using multidisciplinary techniques, we have been using the brain of a tropical fish, the dwarf gourami (*Colisa lalia*), because the GnRH neurons of this fish are large (20 ~ 30 μm in diameter) and make a tight cell cluster without intercalating glial cells just beneath the meningeal membrane so that one can readily record the activities of a single GnRH neuron in a whole brain in vitro preparation using sharp microelectrodes as well as patch pipettes under visual guidance. Thus, it is possible to study, in a semi-intact whole brain, the physiology, morphology, and cell biology of single GnRH neurons by taking advantage of the in vitro experimental conditions. Because the GnRH neuronal cell bodies in most vertebrate species are small (about 10 μm in diameter) and diffusely distributed in various brain regions, it has been extremely difficult to study various features of single GnRH neurons (Kelly et al., 1984). Therefore, the dwarf gourami clearly has experimental advantages over other vertebrates to study the cellular physiology and morphology of GnRH neurons. Although recent technological development has enabled electrical recording of GFP-labeled hypothalamic GnRH

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neurons visualized in transgenic mice brain slices (Skynner et al., 1999; Spergel et al., 1999; Suter et al., 2000a,b), this necessitates a laborious preparation and does not seem to be suitable for stable recording. In the dwarf gourami, we have also found out that the TN-GnRH neurons exhibit exocytotic peptide release activity even from the cell bodies (Oka and Ichikawa, 1991, 1992; Ishizaki and Oka, unpublished data); this is of great advantage because the peptides are usually released from small nerve terminals or varicosities, which are very difficult to access for direct measurements of their electrical or release activities. Thus, we believe that the whole brain in vitro preparation of the dwarf gourami is ideal for studying the molecular and cellular mechanisms of peptidergic neuromodulation as well as exocytotic peptide release mechanisms in general. Last but not least, the dwarf gouramis are tropical fish and it is very easy to get sexually mature fish and observe the characteristic reproductive behaviors throughout the year; this is advantageous for the neuroethological analysis, which is essential to know the biological significance of the peptidergic neuromodulation. In this chapter, I will summarize our recent research progress in the physiology and release activity of GnRH neurons, especially focusing on the terminal nerve (TN) GnRH system.

Terminal nerve (TN)–GnRH system, and the diversity of GnRH peptides and neuronal systems

Specific antibodies against various neurotransmitter substances and peptide hormones and highly sensitive immunocytochemical techniques have become available in 1970's, and these enabled us to identify not only the precise location of neurons that produce transmitters and hormones but also the trajectories of their neurites in detail. Owing to these technical advancements, the catalogue of GnRH neuronal systems of vertebrates has been expanding to cover various vertebrate phyla from cyclostomes to mammals including humans (see Silverman et al., 1994). These immunocytochemical studies suggested the presence of extrahypothalamic GnRH systems other than the conventional hypothalamic hypophysiotropic (septo-preoptico-infundibular) system. Schwanzel-Fukuda and Silverman (1980) first reported that the rostral part of such extrahypothalamic GnRH cell groups

belongs to the terminal nerve (TN). The TN was first described anatomically as the last (terminal), and the supernumerary, macroscopically identifiable cranial nerve number zero in elasmobranchs by Fritsch (see Demski and Schwanzel-Fukuda, 1987). The name '*nervus terminalis*' seems to have derived from the association of a portion of this nerve with the rostral end of the lamina terminalis. Although the TN was subsequently identified in various other vertebrates including human embryos, teleosts, urodele amphibians, and so on, very little attention had been paid to it until the immunocytochemical report of Schwanzel-Fukuda and Silverman (1980). Shortly after this report, Demski and Northcutt (1983) and Springer (1983) reported on very exciting findings about the anatomy and function of the TN of the goldfish (*Carassius auratus*). Demski and Northcutt (1983) reported, by using the HRP (horse-radish peroxidase; they did not use GnRH immunohistochemistry) tract tracing method, that the TN cells which are located at the rostral base of the olfactory bulb project to the retina, supracommissural part of the area ventralis of the telencephalon (Vs) and the preoptic area. They could elicit sperm release by electrical stimulation of the optic nerve, which they supposed should antidromically stimulate the retinopetal TN fibers and lead to the collateral activation of the preoptic area and Vs. It had been suggested in teleosts that the preoptic area and Vs are important for the facilitation of sexual behaviors (Koyama et al., 1984; Satou et al., 1984). Putting these pieces of evidence together, they proposed a very adventurous hypothesis about the function of the terminal nerve; the terminal nerve is a new chemosensory system in vertebrates, and the pheromonal stimulation facilitates sexual behavior via its projection to the preoptic area. However, this possibility was later doubted by Fujita et al. (1991). They stimulated the olfactory epithelium of the goldfish with identified sex pheromones (17 α ,20 β -dihydroxy-4-pregnen-3-one and prostaglandin F2 α) and extracellularly recorded the spontaneous activity of the TN cell and the mitral cell (the principal neuron in the olfactory bulb which receives synaptic inputs from the olfactory receptor cells and projects to the secondary olfactory center) of the olfactory bulb. Whereas the mitral cells responded to the pheromonal stimulation, the TN cells did not.

Thus, it is least probable that the TN-GnRH system is chemosensory. We have also shown behaviorally that the total lesion of bilateral TN-GnRH neurons in the male dwarf gourami did not interfere with the overall performance of the sexual behaviors, although it affected the motivational or arousal state of the fish (Yamamoto et al., 1997; also see below). Interestingly, it has been found in some species that the TN-GnRH cell neurites are present in the olfactory nerve and can be traced to the lamina propria of the olfactory epithelium (Eisthen et al., 2000; Wirsig-Wiechmann and Oka, 2002). Furthermore, Eisthen et al. (2000) found that GnRH peptide can modulate the sensitivity of olfactory receptor neurons via modulation of inward Na^+ channels and outward K^+ channels. Demski and Northcutt (1983) may have labeled these neurites retrogradely from the olfactory epithelium. On the other hand, Springer (1983) reported on the anatomy of the goldfish TN including its retinopetal projection by using the cobalt chloride tract-tracing method. Subsequent immunohistochemical studies have clearly demonstrated that these retinopetal projections of TN are GnRH-immunoreactive (Münz and Stumpf, 1981; Stell et al., 1984; Oka and Ichikawa, 1990; Uchiyama, 1990). The presence of GnRH-immunoreactive retinopetal projection proved to be general anatomical characteristics of teleost TN-GnRH systems. Interestingly, the GnRH-immunoreactive retinopetal projection system seems to be unique to teleosts among vertebrate species. With the exception of the retinopetal projections, the presence of GnRH-immunoreactive TN system has been recognized in all vertebrate species reported to date (Demski and Schwanzel-Fukuda, 1987).

In addition to the TN-GnRH system, another extrahypothalamic GnRH system, with their cell bodies in the midbrain and their axons projecting widely in the caudal part of the brain, has been reported in various vertebrate species. For example, in elasmobranchs, teleosts, amphibians, reptiles, birds, and primitive mammals, midbrain GnRH neuronal system has been reported, all of which are immunoreactive to chicken II GnRH (see below). On the other hand, TN and POA-GnRH neurons seem to express various forms of GnRH according to the species. Therefore, the midbrain GnRH neuronal system may be the most evolutionally conservative one (Muske,

1997; Sherwood et al., 1997). From the anatomical similarities (location of cell bodies and their projection routes) between the chicken II-ir midbrain GnRH neurons and the nucleus of the fasciculus medialis longitudinalis (nFLM), some authors have described them as identical. However, Yamamoto et al. (1998a) have examined this possibility experimentally by double-labeling experiments, in which the nFLM and their descending axons were labeled by retrograde transport of biocytin injected into the spinal cord, and the GnRH-ir profiles were labeled by chicken II specific antibody. As a result, they found that, although the two systems are anatomically juxtaposed with each other, they are separate entities. The cell bodies of nFLM are large and possess thick and straight spinal descending axons, while the midbrain chicken II-GnRH neurons also have large cell bodies but have thin and tortuous axons with many branches.

Although the function of hypothalamic GnRH system is clear, the GnRH neuronal cell bodies in most vertebrate species are small and diffusely distributed in various brain regions, and their axons do not usually form distinct bundles that lead to the median eminence, where GnRH peptides are supposed to be released from the axon terminals into the portal vessels and transported to the gonadotropes to stimulate the release of gonadotropins. Therefore, it has been and still is very difficult to identify the cells of origins of the hypophysiotropic GnRH neurons. Teleosts lack the structure that is equivalent to the median eminence, and the axons of all the hypophysiotropic hormone-producing cells project directly to the pituitary gland. Interestingly, in some teleosts including the dwarf gourami, hypophysiotropic POA-GnRH neurons make distinctive cell clusters in the preoptic area and project their axons to the pituitary, forming a robust axon bundle (Oka and Ichikawa, 1990). Furthermore, we found that, about two weeks after bilateral electrolytic lesions in the TN-GnRH neurons, the POA-GnRH neurons and their axonal projections to the pituitary could be observed in isolation (Yamamoto et al., 1995, 1997). By taking advantage of the whole brain *in vitro* preparation of the dwarf gourami, injection of axonal tracers such as neurobiotin or DiI into the pituitary became much easier compared to that *in vivo*, and the neurons of origins that project to the pituitary could be

retrogradely labeled, and the double-labeling with GnRH immunocytochemistry clearly demonstrated that the POA-GnRH neurons are the main population of GnRH neurons that project to the pituitary and thus can function actually as a gonadotropin-releasing hormone system (Yamamoto et al., 1998b).

In addition to the studies of anatomically different GnRH neuronal systems, there have been another series of studies aimed at clarifying the diversity and evolution of various molecular forms of GnRH peptides (reviewed by Sherwood et al., 1997). GnRH is composed of only ten amino acid residues, but some of them are variable and more than fourteen (Montaner et al., 2001; the number of variants are still increasing) different molecular species of GnRH peptides have been sequenced thus far (for reviews, see Sherwood et al., 1993, 1997). Moreover, in most classes of vertebrates, several types of GnRH have been found in the brain of single species. For example, salmon GnRH, seabream GnRH, and chicken II GnRH have been found in the many teleost brains. Even in humans, where the mammalian GnRH (originally identified LHRH) has been regarded as one and the only type of GnRH peptide in the brain, expression of a second gene for GnRH that is equivalent to chicken II GnRH was reported (White et al., 1998). They further classified GnRH peptides into GnRH I to III and discussed the evolutionary relationships among them. According to them, GnRH I contains releasing forms produced in the hypothalamus, GnRH II contains evolutionarily conservative mesencephalic forms, and GnRH III contains telencephalic forms that are generally found in the terminal nerve system. They suggest that these three types of GnRH peptides belong to evolutionarily distinct GnRH groups. As discussed later, GnRH have long been suggested to be responsible for the generation of long slow EPSP by inhibiting K^+ M current in the sympathetic ganglion cells of the bullfrog. Interestingly, it has been reported that GnRH II (chicken II GnRH) is expressed in the frog sympathetic ganglia (Troskie et al., 1998). They further found a GnRH receptor selective for chicken II GnRH in the ganglia, and suggested that endogenous chicken II GnRH may play a role in synaptic transmission (or neuromodulation of K^+ M current) in the sympathetic ganglia via a receptor specific for chicken II GnRH.

The anatomical correspondence between the morphologically as well as functionally divergent multiple GnRH systems and a variety of molecular species of GnRH peptides had been rather difficult to demonstrate experimentally until recently. This is mainly because the peptidergic neurons are generally small and scattered in the brain and are hard to label with axonal tracers or make local brain lesions to allow the specific group of GnRH neurons to degenerate. For this kind of analysis, it has proved to be much more advantageous to use the teleost brains than to use the mammalian or other vertebrate brains (see Kim et al., 1997; Oka, 1997; Parhar, 1997). Thus, correspondence between the anatomy of GnRH system and the molecular species has been clearly demonstrated experimentally in the dwarf gourami (Yamamoto et al., 1995, 1997; Ishizaki et al., 2002). As already mentioned, the TN-GnRH neurons of the dwarf gourami make tight cell clusters on both sides of the brain in the transitional area between the olfactory bulb and the telencephalon. Therefore, it is possible to make local electrolytic brain lesions in the TN-GnRH neuron clusters (ganglion of the terminal nerve) on both sides of the brain and make them degenerate. By combining local brain lesioning with immunohistochemistry using antibodies specific to different GnRH molecules, it became possible to differentiate the projection areas of multiple GnRH neuronal systems. Because of the technical difficulty of selectively lesioning specific group of GnRH neurons, comparable anatomical studies have not been carried out in other vertebrate brains. However, recent progress in molecular biological techniques have enabled cloning and sequencing genes coding different molecular variety of GnRH peptides, and it became possible to demonstrate the distribution of neurons expressing mRNAs specific to each type of GnRH molecule by using in situ hybridization techniques (Parhar, 1997). Thus, a growing body of evidence suggests the presence of at least three anatomically and functionally different GnRH systems throughout the vertebrate species (Fig. 1), although the cyclostomes seem to be exceptional (Sower, 1997). For example, in teleosts such as dwarf gourami (Yamamoto et al., 1995; Ishizaki et al., 2002), tilapia (Parhar, 1997), and seabream (Okuzawa et al., 1997), general agreement has been reached, by using specific

Multiple GnRH systems

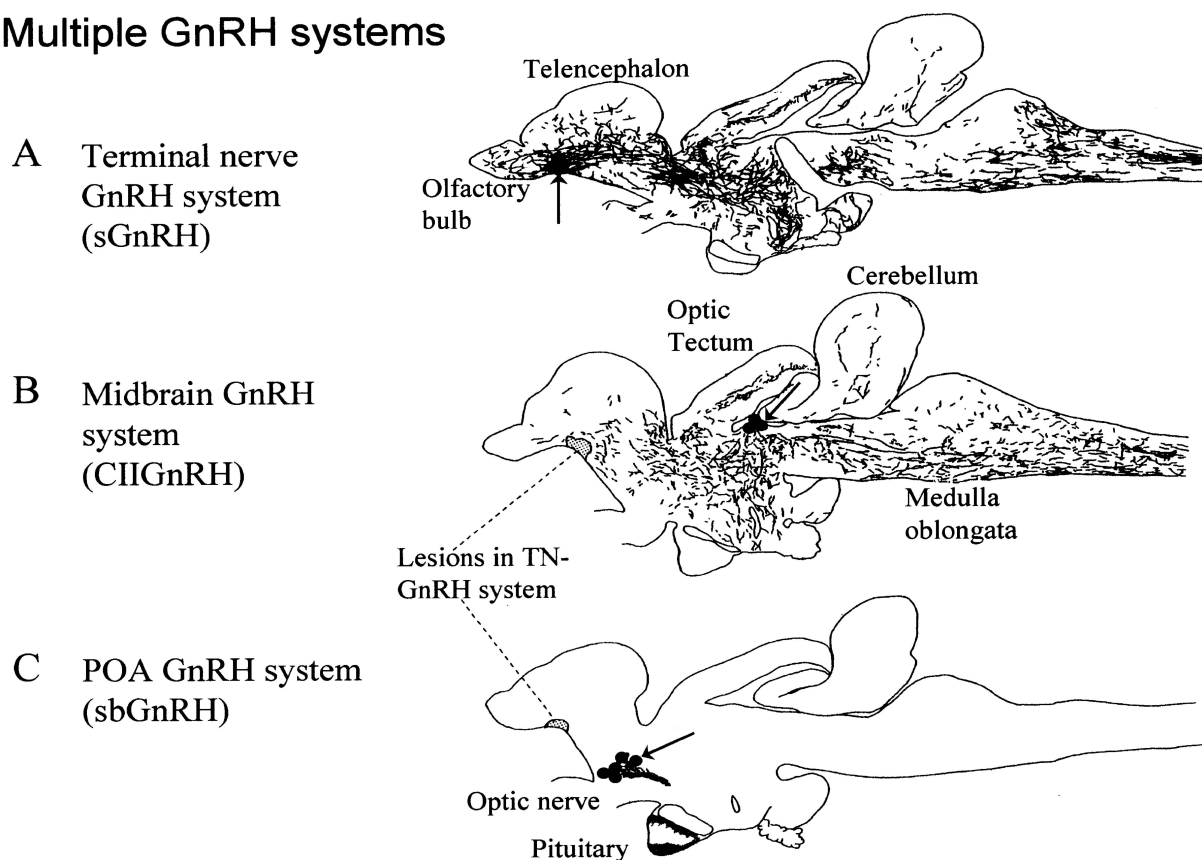


Fig. 1. Schematic illustrations of the distribution of cell bodies and fibers of the TN- (A), midbrain- (B), and POA-GnRH (C) systems, based on the combined local brain lesioning and immunocytochemistry using antisera specific for different molecular species of GnRH. It is now known that each system is immunoreactive to sGnRH, cIIGnRH, and sbGnRH, respectively. The arrows indicate the location of cell bodies, and the stippled areas indicate brain lesions in the TN-GnRH cell bodies. (Modified from Yamamoto et al., 1995.)

immunohistochemistry and in situ hybridization, that the TN-GnRH system expresses salmon GnRH, the POA-GnRH system seabream GnRH, and the midbrain GnRH system chicken II GnRH. From these and other lines of evidence, we suggest that there are basically three different GnRH neuronal systems with different anatomy, molecular species, and functions. (1) The POA (preoptic, hypothalamo-hypophysial)-GnRH system (Fig. 1C) that projects almost exclusively to the median eminence (directly to the pituitary gland in the case of teleosts) but very sparsely to the brain. (2) The TN-GnRH system (Fig. 1A) that projects widely throughout the brain from the olfactory bulb to the spinal cord but never to the pituitary gland (Oka and Ichikawa, 1990; Oka and Matsushima, 1993; Yamamoto et al.,

1995). From this anatomical feature, the TN-GnRH system should not be directly involved in the control of pituitary functions. Instead, it is suggested to function as an important neuromodulatory system (see below, and Oka, 1997; Oka and Abe, 2002). The finding in the goldfish that disruption of TN-GnRH system does not affect the ovarian development or ovulation (Kobayashi et al., 1994; Kim et al., 2001) strongly supports this. (3) The midbrain GnRH system (Fig. 1B) that is considered to be the most evolutionarily conservative GnRH system in the vertebrates. All the midbrain GnRH systems reported thus far are immunoreactive to chicken II type of GnRH peptide, and they also project widely in the brain, especially in the caudal brain structures, but never to the pituitary gland, and thus seems to have

some neuromodulatory functions similar to the TN-GnRH system.

Here we would like to stress that it would not be a fruitful discussion to correlate specific molecular species of GnRH peptide or GnRH receptor types with specific functions as has been frequently done, since the relationship between the molecular species and the anatomy of GnRH system varies according to the animal species except for the conservative linkage between the midbrain GnRH system and the chicken II GnRH (see above). Furthermore, the ligand–receptor relationships in GnRH neuronal systems do not seem to be so specific so that it often happens that the GnRH species that should not be considered as physiologically active peptide, as judged from its projection pattern, can often evoke effective responses when administered exogenously in vivo or in vitro (King and Millar, 1997). Therefore, we strongly argue that it is not the type of GnRH molecule or GnRH receptors that determine the function of the particular GnRH neuronal system but the specific projection pattern of the GnRH neurons, i.e., the projection pattern or the distribution areas of neurites of each GnRH neuronal system.

Recent findings on the GnRH neuronal systems in the ancestral chordates, ascidians, are noteworthy from the evolutionary viewpoint of the multiple GnRH neuronal systems of vertebrates. The presence of GnRH-like substance was first reported by immunocytochemical studies using vertebrate GnRH antisera (Georges and Dubois, 1980; Mackie, 1995). We made a precise mapping of GnRH-immunoreactive neurons in the central as well as in peripheral nervous systems of *Ciona* (Tsutsui et al., 1998). We carried out immunohistochemistry using anti-salmon GnRH and anti-chicken II GnRH antisera and found GnRH-immunoreactive structures in the neural ganglion, which is considered to be the central nervous system of ascidians, and along the inner wall of the dorsal blood sinus, which is a thick blood cavity running from the cerebral ganglion towards the gonads along the gonoducts (spermiduct and oviduct; the ascidians are hermaphrodites), as well as on the surface of the ovary. Although the existence of pituitary homologs and the hypothalamo–hypophysial–gonadal axis in the ascidians is still controversial, such findings of ascidian GnRH neuronal system may shed light on the evolution of

vertebrate GnRH nervous system. There are some recent interesting findings on the ascidian GnRH molecules. Powell et al. (1996) reported two new molecular species of GnRH that are specifically produced in the nervous system of ascidians (Tunicate I and Tunicate II). Di Fiore et al. (2000) found mammalian GnRH and chicken I GnRH in the gonad of the tunicate, *Ciona*, and showed that these GnRH peptides facilitate synthesis and release of sex steroids in the gonad of *Ciona* as well as release of LH from the rat pituitary. Both of these GnRH peptides are expressed in the hypophysiotropic POA GnRH neurons, and it is interesting that this type of GnRH peptides already existed in the ancestral chordate, ascidians. These results led them to conclude that the amino acid sequence and function of these two molecular forms of GnRH peptides have been well conserved during evolution of chordates.

Morphology and physiology of single GnRH neurons

Because of the technical difficulties of recording from single GnRH neurons, there has been very limited information on the morphological and physiological characteristics of individual GnRH neurons. By taking advantage of the accessibility of single GnRH neurons in the whole brain in vitro preparation of the dwarf gourami, we succeeded in recording and labeling single GnRH neurons intracellularly (Oka, 1992; Oka and Matsushima, 1993). The morphology and physiology of single GnRH neurons have already been well described in detail (see Oka, 1997 for review), and these will not be repeated here in detail. In summary, most of the TN-GnRH cells showed endogenous slow (1–7 Hz) regular pacemaker potentials (Fig. 2). The intrinsic nature of this activity was demonstrated by the voltage dependence of the pacemaker frequency, rhythm resetting, and persistence of rhythmicity after synaptic isolation. Only a small number of TN cells showed either irregular or bursting discharge patterns. Anatomical observation of intracellularly labeled cells (Fig. 3) showed that, regardless of discharge patterns, all the TN cells had multiple axonal branches which project to those areas where we had previously demonstrated dense GnRH-immunoreactive fibers (Oka and Ichikawa, 1990; Yamamoto et al., 1995). In-

Intracellular recording of TN-GnRH neurons

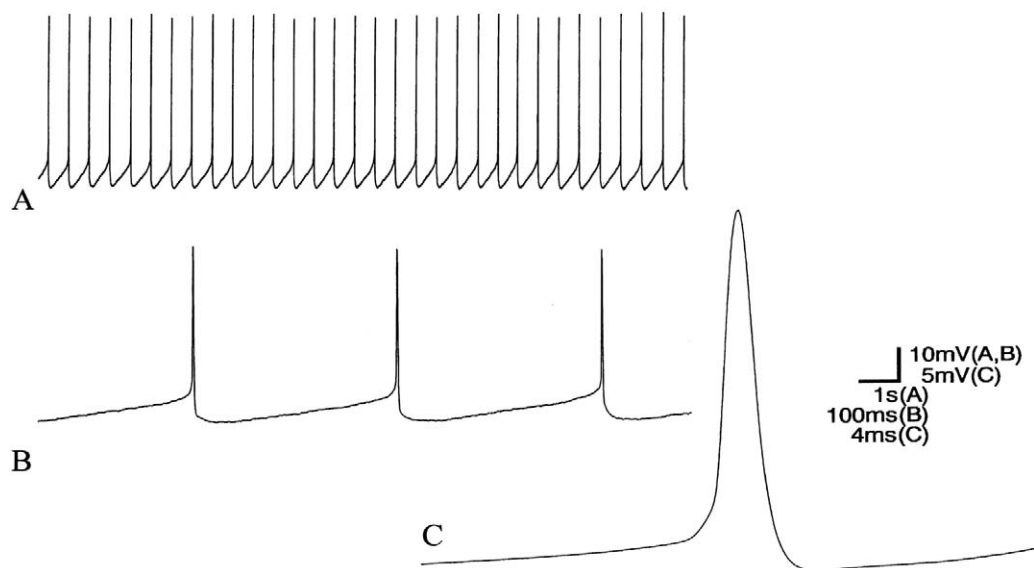


Fig. 2. Intracellular recordings on different time scales of spontaneous regular pacemaker activities of TN-GnRH cells in a whole brain in vitro preparation. It is characterized by regular and slow-frequency spikes with rather long durations. During interspike intervals, smooth and gradual depolarizations, which are similar to the cardiac pacemaker potentials, are evident. (Modified from Oka and Matsushima, 1993.)

terestingly, in the recordings where several different cells were recorded from the same preparations, they showed similar pacemaker frequencies. Therefore, it was suggested that the frequencies of pacemaker activities are related to the physiological conditions of the fish, which are controlled by the hormonal or environmental factors. Furthermore, the morphological features of TN-GnRH neurons are relevant for the regulation of excitability and/or transmitter release (see below) of target neurons in a wide variety of brain regions simultaneously via multiple axonal branches (Fig. 3). Thus, TN-GnRH cells possess the morphological and physiological characteristics relevant to function as a neuromodulator.

Mechanisms of generation and modulation of pacemaker potentials

From the results thus far obtained, the pacemaker potential and its frequency or pattern seems to be important for TN-GnRH neurons as a neuromodulator. Therefore, we focused on the generation and modulation mechanisms of pacemaker frequencies (Oka, 1995, 1996; Abe and Oka, 1999, 2000, 2002).

We first found a novel TTX-resistant persistent Na^+ current, $I_{\text{Na}(\text{slow})}$, which supplies the persistent depolarizing drive and plays an important role in the generation of pacemaker potentials in TN-GnRH cells (Oka, 1995). This current was discovered when the pacemaker potentials were resistant to tetrodotoxin (TTX) but were readily blocked by substituting Na^+ -impermeant ions (tetramethylammonium or choline) for Na^+ in the perfusing solution, and the resting membrane potential became more hyperpolarized than the control level. $I_{\text{Na}(\text{slow})}$ current was further characterized by using the patch voltage clamp recording (Oka, 1996). $I_{\text{Na}(\text{slow})}$ currents could be isolated pharmacologically by blocking K^+ currents, Ca^{2+} currents, and conventional fast Na^+ current. The current was characterized by resistance to TTX blockade, dependence on external Na^+ , slow activation, very slow and little inactivation, and wide overlap of activation and inactivation curves (window currents) near the resting potential. These characteristics are distinct from those of conventional fast Na^+ current, and are relevant for the generation of persistent inward currents necessary for the pacemaker activity of TN-GnRH cells.

Intracellular staining of TN-GnRH neurons; reconstruction from serial sections

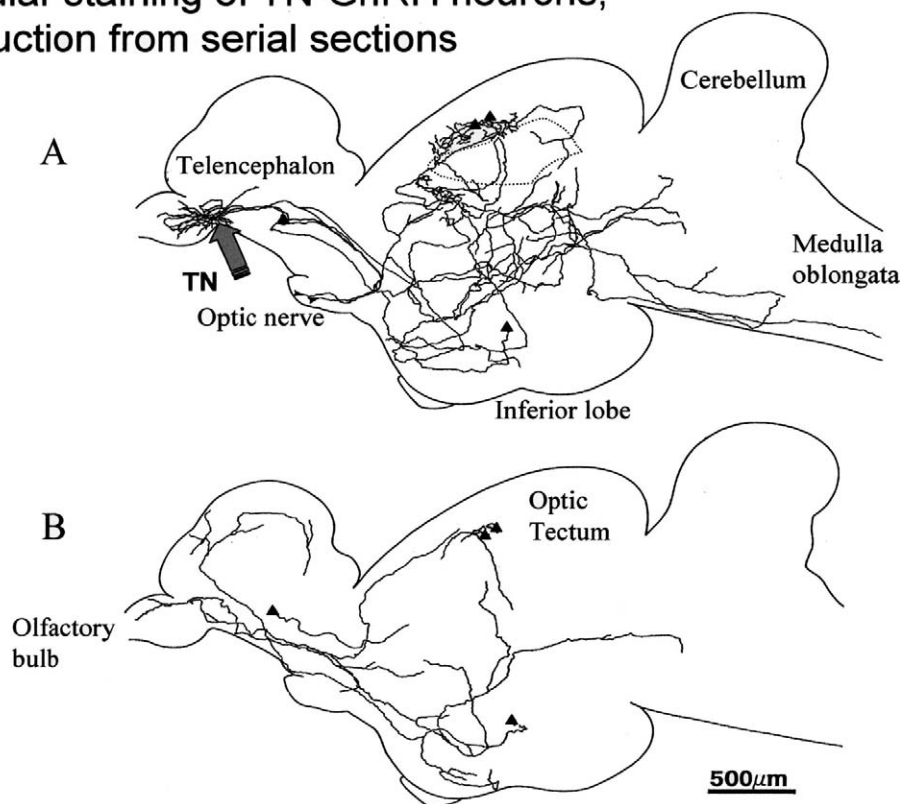


Fig. 3. Illustrations of an intracellularly labeled single TN-GnRH cell reconstructed from serial sagittal sections and are drawn on a representative parasagittal plane. Biocytin was injected into a single neuron unilaterally (the arrow in A). Labeled neurites are seen widely in the brain from the olfactory bulb to the medulla and the rostral spinal cord, and from the medial to the lateral ends. In addition, some axons cross to the contralateral side (B) at various levels, e.g., in the anterior commissure, posterior commissure, tectal commissure, and ventral tegmentum (filled triangles), and project widely on the contralateral side. (Modified from Oka and Matsushima, 1993.)

We next searched for the candidate outward currents that should counteract the persistent depolarizing drive supplied by $I_{Na(slow)}$. We demonstrated, by using the whole-cell voltage clamp recording, that the TN-GnRH cells have at least four types of voltage-dependent K^+ currents; (1) 4-aminopyridine (4AP)-sensitive K^+ current, (2) Tetraethylammonium (TEA)-sensitive K^+ current, and (3), (4) two types of TEA- and 4AP-resistant K^+ currents. Among these, the second, TEA-sensitive K^+ current evoked from a holding potential of -100 mV was slowly activating, long-lasting and showed comparatively low-threshold of activation. This current was only partially inactivated at steady state of -60 to -40 mV, which is equivalent to the resting membrane potential of the TN-GnRH neurons. Furthermore, in current-

clamp recordings bath application of TEA together with TTX reversibly blocked the pacemaker potentials. Therefore, we concluded that the TEA-sensitive K^+ current, $I_{K(V)}$, is the most likely candidate that contributes to the repolarizing phase of the pacemaker potentials of TN-GnRH cells.

From these results, we concluded that the basic pattern of pacemaker activities of the TN-GnRH neurons, especially their subthreshold component, is generated in the following manner. When the TN-GnRH cells are at a resting potential, considerable amount of $I_{Na(slow)}$ is non-inactivated and supplies the persistent depolarizing drive, and the membrane potentials gradually depolarizes. When the membrane potential reaches the activation threshold for the $I_{K(V)}$, outward current develops, and

the net flux of current reverses to outward. Then, the membrane potential becomes hyperpolarized and deactivates the K^+ current, and the next cycle begins. In addition, we have recently found out that the voltage-dependent N-type Ca^{2+} current sensitive to ω -Conotoxin GVIA and the voltage-independent Ca^{2+} influx sensitive to Zn^{2+} or SK&F96365 (store-operated Ca^{2+} current, SOC) may be also involved in the generation of pacemaker potentials (Abe and Oka, 2002; Oya et al., 2001). Considering the activation kinetics, the N-type Ca^{2+} current are suggested to be involved in the action potential phase (suprathreshold phase) of the pacemaker activities, together with the TTX-sensitive conventional $I_{Na(fast)}$. The SOC probably contributes to the generation of background persistent inward current together with $I_{Na(slow)}$.

We next searched for possible candidates that modulate the pacemaker activity of GnRH neurons (Abe and Oka, 2000). We found that the salmon GnRH (sGnRH), which is the same molecular species of GnRH produced by TN-GnRH neurons themselves, affects the pacemaker activities as follows (Fig. 4).

In Ringer solution, TN-GnRH neuron showed slow regular beating discharge. During the bath application of sGnRH, the firing frequency of pacemaker activity was transiently decreased (early phase), and subsequently increased (late phase). It should be noted that this modulation occurred without detectable membrane potential changes in most cases, which precludes the possibility that the modulation of pacemaker activity, decrease and then increase of frequency, may be simply caused by hyperpolarization and depolarization of the membrane, respectively. This biphasic modulation of pacemaker activity was also evoked by bath application of another kind of GnRH peptide (mammalian GnRH) but was not evoked by inactive GnRH analog (GnRH antagonist alone) and was inhibited or attenuated by GnRH antagonist. These results strongly suggested that modulation by GnRH peptide of pacemaker activity of TN-GnRH neurons is caused by GnRH receptor activation, although there does not seem to be a selectivity of different molecular species of GnRH for the receptor activation. This is in agreement with the report that GnRH receptors of non-mammalian species respond to any types of

GnRH peptides (King and Millar, 1997). This does not, however, mean that GnRH released from the GnRH neurons that belong to the other GnRH systems (other molecular species of GnRH) activates the TN-GnRH neurons under physiological conditions, because immunoreactive fibers of the other GnRH systems are not distributed near the TN-GnRH neurons (Yamamoto et al., 1995).

In order to elucidate possible mechanisms underlying this modulation of pacemaker activity, we next examined whether a G-protein-coupled process was involved in this modulation. There are alternative possible mechanisms to explain the biphasic modulations of the frequency of pacemaker activity of TN-GnRH neurons. First, GnRH receptors may exist on the cell surface of TN-GnRH neurons, and the pacemaker activity of TN-GnRH neurons may be directly modulated by the downstream cell signaling pathways. Second, GnRH receptors may exist on the cell surface of non-GnRH neurons, and the pacemaker activity of TN-GnRH neurons may be indirectly modulated by these neurons. To test these alternative possibilities, we dialyzed the cell with GDP- β -S, a GDP derivative that is a competitive inhibitor of many G-protein-mediated processes, by including it in the patch pipette solution, since it has been already established that the GnRH receptors are members of the G-protein-coupled receptors (Stojilkovic et al., 1994a). After the diffusion of GDP- β -S into the TN-GnRH neuron, bath application of sGnRH failed to evoke any modulation of the firing frequency of pacemaker activity. From these results, we suggested that G-protein-coupled process mediates this biphasic modulation of the pacemaker activity by sGnRH in TN-GnRH neurons. It has been reported that GT1-7 cells express GnRH receptors (Krsmanovic et al., 1993; Stojilkovic et al., 1994a,b). Moreover, GnRH neurons of hypothalamic culture have been shown by double immunostaining to co-express GnRH and GnRH receptors (Krsmanovic et al., 1999). Taken together, it is highly possible that the GnRH receptor exists on the cell surface of TN-GnRH neurons and plays a triggering role in modulating the ion channel(s) underlying the pacemaker activity via G-protein-mediated signaling pathways. To confirm this possibility, the molecular nature of the GnRH receptors expressed on the cell surface of TN-GnRH neurons should be identified by using in

Biphasic modulation of pacemaker potentials by sGnRH

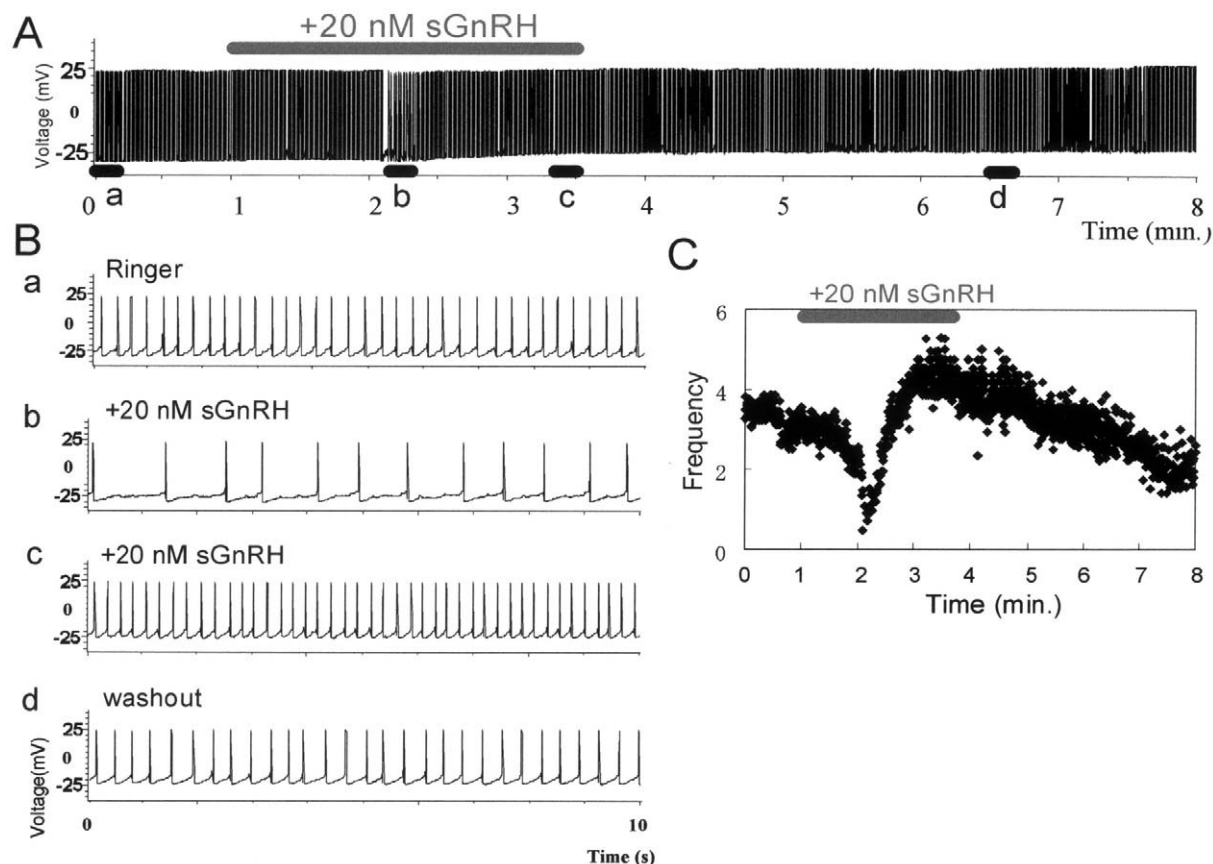


Fig. 4. Modulation of pacemaker frequency by sGnRH. (A) In a current-clamp whole cell recording from a TN-GnRH neuron, bath application of sGnRH, the same molecular species of GnRH peptide produced by TN-GnRH neurons themselves, biphasically modulated their pacemaker activity. (B) Bath application of sGnRH transiently decreased (b) and subsequently increased the frequency of pacemaker activity (c). Following washout, the firing frequency of pacemaker activity recovered (d). (C) Frequency of pacemaker activity plotted against the time course. (Modified from Abe and Oka, 2000.)

situ hybridization and patch-RT-PCR methods in the future study.

Since the basic rhythm of the pacemaker potential was found to be generated by the interplay of $I_{Na(slow)}$ and $I_{K(V)}$ currents (see above), we expected that sGnRH may modulate $I_{Na(slow)}$ or $I_{K(V)}$ or both. However, our preliminary experiments showed that the bath application of sGnRH failed to evoke any noticeable modulation of the $I_{Na(slow)}$ or $I_{K(V)}$. Therefore, we suspected that Ca^{2+} currents that are suggested to be also involved in the generation of pacemaker activities (see above) may be modulated for the facilitation of pacemaker potentials, and so

we examined such possibilities (Abe and Oka, 2002; Oya et al., 2001). We used several specific blockers for each type of voltage-dependent Ca^{2+} currents (Nifedipine for L-type, ω -Conotoxin GVIA for N-type, and ω -Agatoxin TK for P/Q type) as well as those for the voltage-independent SOC (Zn^{2+} and SK&F96365). We found that the N-type Ca^{2+} currents and the SOC are involved in the facilitation of pacemaker potentials by sGnRH (Oya et al., 2001).

Then we isolated Ca^{2+} currents by the whole cell voltage clamp recording of TN-GnRH neurons (Abe and Oka, 2002). We could identify at least two kinds of Ca^{2+} currents; transient low voltage-

Effects of sGnRH on Ca^{2+} currents in TN-GnRH neurons

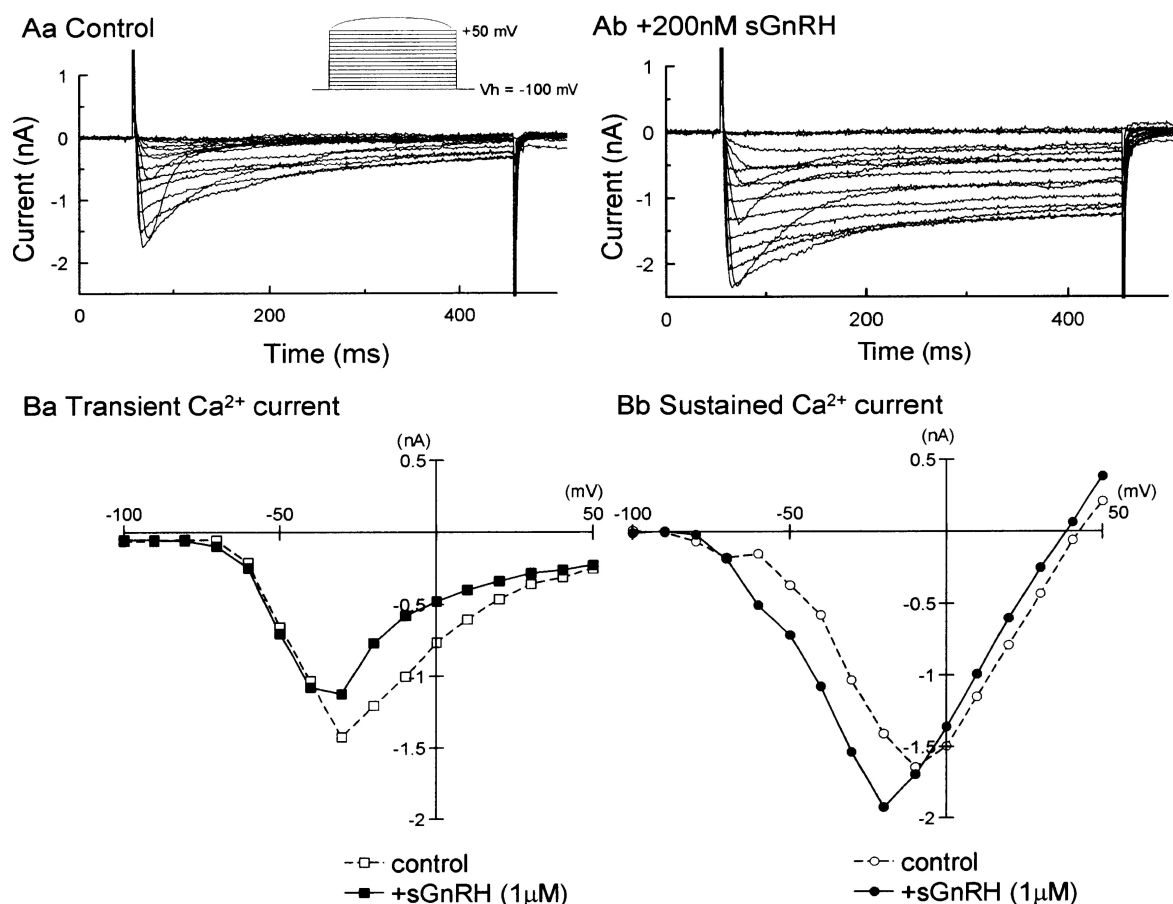


Fig. 5. Effects of sGnRH on Ca^{2+} currents in TN-GnRH neurons. (A) Isolated Ca^{2+} current responses elicited from a holding potential of -100 mV before (a) and after the addition of 200 nM sGnRH to the bath solutions (b). (B) I/V curves were constructed by plotting the averaged current amplitudes ($n = 10$) evoked from holding potentials of -100 mV before and after the addition of 200 nM sGnRH to the bath solution. The current-voltage relations of transient current (a) and sustained current (b) are shown. The error bars are not indicated for clarity. (Modified from Abe and Oka, 2002.)

activated (LVA) currents and persistent high voltage-activated (HVA) currents. When we applied sGnRH to the perfusing solution, we found that the persistent HVA current is affected by sGnRH. The activation of HVA current shifted to more hyperpolarized potentials, and the current amplitude was also increased by sGnRH (Fig. 5). From these results, we suggested that a kind of HVA Ca^{2+} current is modulated (facilitated) by sGnRH and contributes to the late-phase increase of pacemaker potentials. Although we have not yet examined the specific Ca^{2+} chan-

nel blockers in the voltage clamp recording, it is highly possible that this HVA Ca^{2+} current is the ω -Conotoxin-sensitive N-type Ca^{2+} current studied in the current clamp recording. We also examined the mechanisms responsible for the early phase decrease of pacemaker potentials by GnRH. Since the ryanodine receptor antagonist Ruthenium Red and IP_3 receptor antagonist heparin introduced into the cell by including them in the pipette solution disrupted the early-phase decrease of pacemaker potentials but left the late-phase increase intact, it was

suggested that the early-phase decrease was brought about through the activation of Ca^{2+} -dependent K^+ currents by Ca^{2+} released from the intracellular stores. This was confirmed by current clamp recording in which apamin, a specific blocker of small conductance Ca^{2+} -dependent K^+ currents, inhibited the early phase decrease of pacemaker potentials (Abe and Oka, 2002). Furthermore, in voltage clamp recordings, we found transient potassium current that is dependent on the presence of external Ca^{2+} ions, which is kinetically different from the 4AP-sensitive potassium A-current that we previously found in this neuron (Abe and Oka, 1999). We assumed that this current corresponds to the apamin-sensitive $\text{I}_{\text{K}(\text{Ca})}$ and checked whether this tentative $\text{I}_{\text{K}(\text{Ca})}$ could be modulated by sGnRH or not. Repetitive 200 ms test pulses were continuously applied to the cell during the recording to evoke the tentative $\text{I}_{\text{K}(\text{Ca})}$, and sGnRH was perfused for certain periods. Although the current showed some degree of rundown during the recording, the current amplitude was clearly increased during the application of sGnRH. Thus, we suggest that this tentative $\text{I}_{\text{K}(\text{Ca})}$ that is facilitated by sGnRH contributes to the early phase transient decrease of frequency of pacemaker potentials.

As we have described so far, the pacemaker potential of TN-GnRH neurons are frequency-modulated by sGnRH biphasically, and the modulation consists of the transient decrease and subsequent increase in the frequency of pacemaker potentials. Such biphasic modulations of the electrical activities have been reported for the changes in membrane potentials of clonal GH3 cell lines induced by TRH (Ozawa and Sand, 1986), and those of gonadotropes and immortalized GnRH cell line (GT1-7 cell) induced by GnRH (Zheng et al., 1997; van Goor et al., 1999). In such cases, it has been suggested that a transient hyperpolarization arises from the activation of Ca^{2+} -activated K^+ currents induced by Ca^{2+} released from the intracellular stores by receptor activation. It is also suggested that Ca^{2+} influx through voltage-independent channels that are activated by depletion of Ca^{2+} stores (SOC) may be activated after this and are involved in the increased pacemaker frequency in the later phase (van Goor et al., 1999). As reported in the adrenal chromaffin cells, this kind of calcium influx may further be involved in the release of GnRH peptides (see Fomina and Nowycky,

1999). Similar mechanisms may exist in TN-GnRH neurons. Future studies should analyze the changes of $[\text{Ca}^{2+}]_i$ induced by GnRH application and analyze the properties of target voltage-dependent and/or voltage-independent channel(s) modulated by the GnRH-induced signaling pathways.

What, then, is the physiological significance of such modulations of pacemaker activity by sGnRH upon TN-GnRH neurons? TN-GnRH neurons of the dwarf gourami make tight cell clusters without intervening glial cells (Oka and Ichikawa, 1990; Oka and Matsushima, 1993; Oka, 1997), and the possibility of active exocytotic release from the cell body and its vicinity (somatodendritic release of GnRH) has been suggested (Oka and Ichikawa, 1991, 1992; also see below). Somatodendritic release of neurohypophysial peptides by exocytosis from the hypothalamic magnocellular neurons has also been reported (Pow and Morris, 1989). On the other hand, there are studies showing that GnRH receptors are widely distributed throughout the brain (Stojilkovic et al., 1994b; Jennes et al., 1997). In addition, considerable overlap of the brain areas that contain GnRH-producing cells and those that exhibit expression of GnRH receptor mRNA, has been reported (Jennes et al., 1996). From these observations and ours, it is suggested that GnRH released from GnRH neurons facilitates the activities of their own (autocrine) and/or neighboring GnRH neurons (paracrine) and may cause synchronized positive feedback facilitation of multiple GnRH neurons. It has been well established that the release of GnRH from the hypothalamus is pulsatile, and this pulsatile release of GnRH is essential for the control of hypothalamo-pituitary-gonadal axis and hence the regulation of normal reproductive function in mammals (Terasawa, 1998; Terasawa et al., 1999). Although the TN-GnRH and POA-GnRH systems may be anatomically and functionally different, it is tempting to assume that the autocrine or paracrine mechanisms may also underlie the pulsatile release of GnRH. It is also known that in oxytocin neurons the release of oxytocin from single neuron into the brain environment stimulates its own activity and thus further release (Freund-Mercier and Richard, 1984; Moos et al., 1984). Therefore, this mechanism is probably common to all neurosecretory neurons or secretory cells, whose synchronized facilitation of firing leads to facilitated release.

Neuromodulatory action of GnRH peptides

Modulation of ion channels by GnRH

We originally proposed the idea that the TN-GnRH system functions as a neuromodulator system in the central nervous system (Oka, 1992; Oka and Matsushima, 1993), from a series of studies using the dwarf gourami TN-GnRH system. However, experimental evidence for a neuromodulatory action of GnRH in the sympathetic ganglia (causing late slow EPSP) dates back to the early 70s (see Jan and Jan, 1983 for review). Jan and Jan reported that GnRH is co-released with Ach from the sympathetic ganglion cells, diffuse for some distance, and evokes late slow EPSP in the postganglionic cells (Jan and Jan, 1983). Later, it was found out that this extremely slow and long-lasting potential change results from the closing of non-inactivating K^+ M current that is active at the resting membrane potential range (Brown, 1988). Although the exact cellular mechanisms of this M current inhibition has yet to be completely understood (Marrion, 1997), its physiological significance is suggested to be the increase in the excitability of the postganglionic neuron membranes. It has been shown that, owing to the membrane potential-clamping effect of the M current, the postganglionic neurons discharge on-to-one action potentials in response to the preganglionic fiber stimulation. During the late slow EPSP, when the M currents are inhibited, the postganglionic neurons now respond with persistent action potential responses.

It has been reported recently that GnRH peptide is also involved in the neuromodulation of Ca^{2+} currents. The Ca^{2+} currents have been classified into L, T, N, P/Q, R types based on the kinetic properties of channel opening-closing and on the specificity of blockage by natural neurotoxins. Among others, the N and P/Q types of Ca^{2+} currents have been reported to be modulated by GnRH (Elmslie et al., 1990), and the signal transduction mechanisms of the modulation have been well studied (Marrion, 1997). Since the N and P/Q type Ca^{2+} channels are localized in presynaptic active zones and are involved in the Ca^{2+} influx necessary to the transmitter release, it may be suggested that GnRH peptide modulates the transmitter release via modulation of these Ca^{2+} currents. However, this has not yet been demon-

strated experimentally and will be an exciting future problem to be examined.

On the other hand, much less information on the possible neuromodulator functions of GnRH is available in the central nervous system. In the rat hippocampus, GnRH induces a long-lasting depolarization associated with increased input resistance (decreased membrane conductance), a decrease in the after-hyperpolarization following a train of action potentials, and a reduction in accommodation of repetitive cell discharge (Wong et al., 1990). However, ion channel modulation and its signal transduction mechanisms in the central nervous system have not been well studied probably because of its technical difficulties compared with the peripheral ganglion neurons. As already mentioned above, we have recently found out that the GnRH peptide produced by the TN-GnRH neurons themselves acts on the TN-GnRH neurons as a neuromodulator to change their ionic channel properties (Abe and Oka, 2000). Since the TN-GnRH neurons project their neurites in wide areas of the brain (Oka and Matsushima, 1993), and the GnRH receptors are also widely distributed in the brain (Stojilkovic et al., 1994b; Jennes et al., 1997), it is suggested that the GnRH peptides released from the neurites in wide areas of the brain may act as neuromodulators via similar mechanisms.

Modulation of neuronal functions by GnRH

We have thus far discussed the neuromodulatory function of GnRH peptides on the ionic channel properties, and now let us discuss the modulation by GnRH peptides of neural functions as a whole. In the teleost retina, which receives dense projection of TN-GnRH fibers arising from TN cells, TN-GnRH fibers are known to synapse on dopaminergic interplexiform cells (Zucker and Dowling, 1987), and GnRH and other neuropeptides affect the retinal ganglion cell activity (Stell et al., 1984; Walker and Stell, 1986). Umino and Dowling (1991) reported that when the retina was superfused with Ringer's solution containing GnRH, horizontal cells depolarized, and their response to small spots increased, whereas their responses to full-field lights decreased. Their results suggested that GnRH acts by stimulating the release of dopamine from inter-

plexiform cells. Furthermore, Behrens et al. (1993) reported that GnRH elicits light adaptive formation of horizontal cell spinules in vitro by stimulating the dopaminergic interplexiform cells. Thus, the TN-GnRH fibers projecting to the retina seem to have a definitive physiological function. It will be an interesting future project to study neuromodulatory action of GnRH on GnRH target cells in the brain (such as olfactory bulb, ventral telencephalon, etc.) and its relation to various spontaneous discharge modes by taking advantage of the whole brain in vitro preparation of the dwarf gourami.

Recently, Eisthen et al. (Eisthen et al., 2000) reported on very interesting results showing that GnRH peptide modulates the sensitivity of olfactory receptor neurons by modulating their Na^+ and K^+ channel properties. They used the olfactory receptor neurons from mudpuppies (*Necturus maculosus*) and did voltage-clamped whole-cell recordings to examine the effects of GnRH on voltage-activated currents in olfactory receptor neurons from epithelial slices. They found that GnRH slowly but reversibly increases the magnitude, but does not alter the kinetics, of a TTX-sensitive inward current (most probably conventional Na^+ current involved in the generation of action potentials) and a certain outward currents. This effect appeared to be seasonal, with more animals responding to GnRH during the courtship and mating season. Taken together, they suggest that GnRH increases the excitability of olfactory receptor neurons and that the terminal nerve functions to modulate the odorant sensitivity of olfactory receptor neurons. Although this hypothesis needs to be further tested by examining the olfactory responses to the odorants and the currents need to be isolated for more rigorous electrophysiological analysis, it will surely provide very attracting further working hypotheses to be tested in the future studies.

Behavioral functions of TN-GnRH system

Unfortunately, we still do not know much about the behavioral consequence of neuromodulation by TN-GnRH system. Wirsig and Leonard (1987) were the first to report effects of terminal nerve lesions on the sexual behaviors of vertebrates. They reported in the male hamster that surgical ablation of the terminal nerve affects male sexual behavior. However, since

they did not use the GnRH immunohistochemistry, the extent of the lesion of the TN-GnRH system as a whole was not clear. We (Yamamoto et al., 1997) took advantage of the morphological features of the dwarf gourami TN-GnRH neurons (the cell bodies are large and make tight cell clusters near the ventral surface of the brain) and examined the behavioral effects of specific and complete lesion of the TN-GnRH system. The sexual behavior of the dwarf gourami consists of several readily quantifiable behavioral repertoire or patterns. We quantified the male sexual behavior patterns during one-hour mating trials. We then placed electrolytic lesions in the bilateral clusters of TN-GnRH neurons of the male fish and allowed them to survive for two weeks. All of the GnRH fibers originating from the TN-GnRH neurons had disappeared when the brains were examined by immunohistochemistry after behavioral tests. When some parameters of sexual behavior patterns before and one/two weeks after the operations were compared, we observed a delicate but characteristic behavioral impairment. The occurrence of one-hour mating trials during which the male failed to perform nest building at all was significantly higher in totally or partially TN-lesioned fish compared to intact, sham-operated, or olfactory nerve-cut controls. This means that they became less motivated to start the whole sequence of behavior. On the other hand, these lesions did not affect the overall incidence of the sexual behavior patterns, once the behavioral sequence was triggered. These results seem to suggest that TN GnRH system is involved in the control of the threshold for the initiation of the nest building behavior, although it is not indispensable for the general performance of reproductive behavior, once the behavioral sequence is triggered. Thus, the TN-GnRH system has a delicate control on the motivational or arousal state of the animal in general. We have recently found out in our preliminary behavioral study that the frequency of the male nest-building behavior remains at an elevated state for three or four hours after the end of an hour of pairing with a sexually mature female partner; the males become highly motivated for the nest-building behavior, and the frequency of this behavior can be used as a highly reliable and reproducible index of motivated state of the fish. By using this behavioral parameter, we are now examining if the activation

of TN-GnRH system is involved in this motivated state.

Fine structural evidence for exocytotic release activity of TN-GnRH neurons

As already described above, the axons of the TN-GnRH neurons are distributed throughout the brain from the olfactory bulb to the spinal cord. Therefore, GnRH peptides should be able to modulate the excitabilities and/or the transmitter release of the target neurons simultaneously via the widely projecting axonal branches, if GnRH peptides are released from these axons. Macromolecules such as peptides are released from the neurons via exocytosis, and we now turn our topic to the exocytotic release of GnRH from the TN-GnRH neurons. The ultrastructure of the TN-GnRH cells has been described by Matsutani and Uchiyama (1986) and Oka and Ichikawa (1991, 1992), and the TN-GnRH neuronal cell bodies have been shown to exhibit characteristics similar to those of other peptide-synthesizing cells, that is, highly indented nucleus, stacks of well-developed rough endoplasmic reticulum and Golgi apparatus, numerous membrane-bound dense-cored vesicles (DCVs), and large electron dense droplets (Matsutani and Uchiyama, 1986; Demski and Fields, 1988; Oka and Ichikawa, 1991). GnRH immunoreactivity has been demonstrated in these dense-cored vesicles in the cell bodies by means of immunoelectron microscopy (Oka and Ichikawa, 1992). Thus, it is suggested that the cell bodies contain releasable GnRH peptides. Interestingly, coated pits or vesicles, which are associated with endocytotic membrane retrieval after an active exocytosis, were frequently distributed beneath the plasma membrane of the cell body and small somatic processes. The fine structural evidence thus suggested a somatodendritic release of GnRH. Furthermore, the cell bodies were closely apposed with each other, and they were closely apposed without intervening glial cells, and there was no structural diffusional barrier. The morphological evidence thus suggested the presence of some kind of paracrine or autocrine activity of GnRH neurons by GnRH peptides. The exocytotic profiles of the DCVs are usually elusive under a conventional EM because of their short time-course. It is to be expected that GnRH peptides released by exocytosis diffuse into

the intercellular space and act on the GnRH neuron itself or the neighboring GnRH neurons.

In the GnRH fiber varicosities, GnRH immunoreactivities have also been demonstrated in DCVs (Oka and Ichikawa, 1992), but none of these structures showed any evidence of the presence of synaptic active zones, that is, postsynaptic densities, widened synaptic clefts, or accumulation of synaptic vesicles. Thus, it may be hypothesized that TN-GnRH cells secrete GnRH non-synaptically from DCV-containing fiber varicosities as well as soma and that it exerts its modulatory action on GnRH receptors located on nearby as well as distant target neurons. Recently, evidence favoring the idea of non-synaptic release of neuropeptides is accumulating (Thureson-Klein and Klein, 1990). Similar mechanisms have also been suggested for GnRH release in the mid-brain central gray of the rat (Buma, 1989). Such mode of peptidergic action may be relevant for long-lasting and wide-spread neuromodulation.

RIA measurement of GnRH release from the brain slices

In order to know the release activity of GnRH peptides from the GnRH systems, we measured the release of GnRH peptides into the medium from the brain–pituitary slices by the radio-immunoassay (RIA) (Ishizaki et al., 2002). To measure the GnRH release activities from different GnRH systems, and to examine whether there are differences between them, we conducted a static incubation of brain–pituitary slices under various conditions, and GnRH released into the incubation medium was measured by RIA. The slices were divided into two parts, the one containing GnRH neurons in the preoptic area and axon terminals in the pituitary (POA-GnRH slices), and the other containing the cell bodies and fibers of TN-GnRH neurons and midbrain tegmentum–GnRH neurons (TN-GnRH slices). We demonstrated that the GnRH release was evoked by high $[K^+]_o$ depolarizing stimuli (in both slices) via Ca^{2+} influx through voltage-gated Ca^{2+} channels. From the results of experiments using specific Ca^{2+} channel blockers, it was suggested that the GnRH release from POA-GnRH slices induced by depolarization is mainly dependent on the Ca^{2+} influx through ω -conotoxin-sensitive N-type Ca^{2+} channels, and

that from TN-GnRH slices induced by depolarization is mainly dependent on both nifedipine-sensitive L- and ω -conotoxin-sensitive N-type Ca^{2+} channels. In either slice, ω -agatoxin-sensitive P/Q type Ca^{2+} channels were not involved. The most prominent difference between the GnRH release from the POA-GnRH and TN-GnRH slices, however, was the presence of sexual difference in the GnRH release only in the POA-GnRH slices. In TN-GnRH slices, our evidence suggested that the store-operated Ca^{2+} influx (SOC) may be involved in the basal (spontaneous and unstimulated) GnRH release. This is interesting, since our recent patch current-clamp studies in TN-GnRH neurons suggest the involvement of SOC in the generation as well as modulation of pacemaker activities of TN-GnRH neurons (Oya et al., 2001; see above). Finally, we found that glutamate application significantly increased the GnRH release in the TN-GnRH but not in the POA-GnRH slices in a dose dependent manner. In accordance with these results, glutamate applications increased the frequency of pacemaker potentials of TN-GnRH cells in a dose dependent manner. Furthermore, in our recent preliminary study, we are accumulating evidence for the existence of ionotropic as well as metabotropic glutamate receptors and their depolarizing effects in TN-GnRH neurons.

New approaches to study GnRH release activities of single GnRH neurons; electrochemistry using carbon fiber electrodes (CFEs)

Although GnRH content in brain and GnRH release from brain slices have been measured with radioimmunoassay (Okuzawa et al., 1990; Yu et al., 1991), it has been difficult to measure GnRH release in real-time. On the other hand, electrochemical techniques using carbon fiber electrodes (CFE) have been recently developed to detect the exocytotic release of neurotransmitters such as catecholamines (Leszczyszyn et al., 1990) and serotonin (Alvarez de Toledo et al., 1993). These techniques can detect electroactive transmitters or hormones released from neurons or secretory cells in real-time. It is also reported that some amino acids such as Trp and Tyr are electroactive (readily oxidizable), and the small peptides that contain these amino acids can be electroactive (Bennett et al., 1981). Because sGnRH

contains three electroactive amino acid residues (two Trp and one Tyr residues), we expected that sGnRH would be electroactive and could be detected by CFE from the GnRH axon terminals in the pituitary (Ishizaki and Oka, 2001).

We devised a CFE by inserting a single carbon fiber of 7 μm diameter into a glass capillary, and the glass capillary was pulled to form a microelectrode by a vertical pipette puller. The carbon fiber was insulated, and the electrode was filled with 2 M KCl and was connected to the patch-clamp amplifier. Having decided the voltage dependence of redox current of synthetic sGnRH solution by a cyclic voltammetry (the oxidation current appeared at the potential higher than 600–700 mV and was maximum at about 900–1000 mV), the holding potential (V_h) of the CFE was set at 900 mV for amperometric recording. sGnRH solution was pressure-ejected to the electrode tip using a puffer pipette, and the oxidation current of sGnRH was recorded. Amperometric currents were recorded in response to the puffer application of sGnRH solution. The amperometric currents could be measured at V_h higher than 600 mV, and they increased up to the voltage limit of the recording system, 1000 mV. The oxidation current peak of sGnRH was in good agreement with those of Trp and Tyr, which are electroactive residues in sGnRH (Bennett et al., 1981; Paras and Kennedy, 1995). Since the oxidation peaks of other electroactive substances (e.g. serotonin at 620 mV and dopamine at 600 mV; Kruk and O'Connor, 1995) are lower than that of sGnRH, we were fairly sure that the current we were measuring was that of sGnRH and not contaminating currents, if the amperometric current recorded at 900 mV disappear at the V_h lower than 600–700 mV.

The dose-response was examined by recording amperometric currents to various concentrations of sGnRH solutions. The V_h was held at 900 mV, and sGnRH was applied by puffer pipette. As a control, Ringer solution was pressure-ejected to the tip of the CFE before each experimental recording, and the control current values were subtracted from each experimental data. The detection limit was between 10^{-5} M and 10^{-6} M in four CFEs tested. Although this concentration may be higher than the physiological serum concentration of GnRH, the local GnRH concentration close to the nerve terminals or release

sites should exceed these concentrations. Therefore, the CFE method described here should be able to detect, in real-time, the release of GnRH in a region very close to the GnRH release sites such as the GnRH axon terminals in the teleost pituitary.

We then used the brain–pituitary slices similar to the one used for the RIA for recording sGnRH release activity by amperometry. It has been reported that the dorsal region of the pituitary contains many GnRH-immunoreactive fibers and terminals originating from POA-GnRH neurons in the dwarf gourami

(Maejima et al., 1994; Yamamoto et al., 1998b). Because the POA-pituitary slices of the dwarf gourami release high amount of GnRH in response to depolarizing stimuli in radioimmunoassays (Ishizaki et al., 2002; see above), it is to be expected that the local concentration of GnRH released from the pituitary will be high enough to be recorded by amperometry. The tip of the CFE was positioned so as to lightly touch the surface of the dorsal region of the pituitary (illustrated in Fig. 6A).

Fig. 6 shows an amperometric current recording

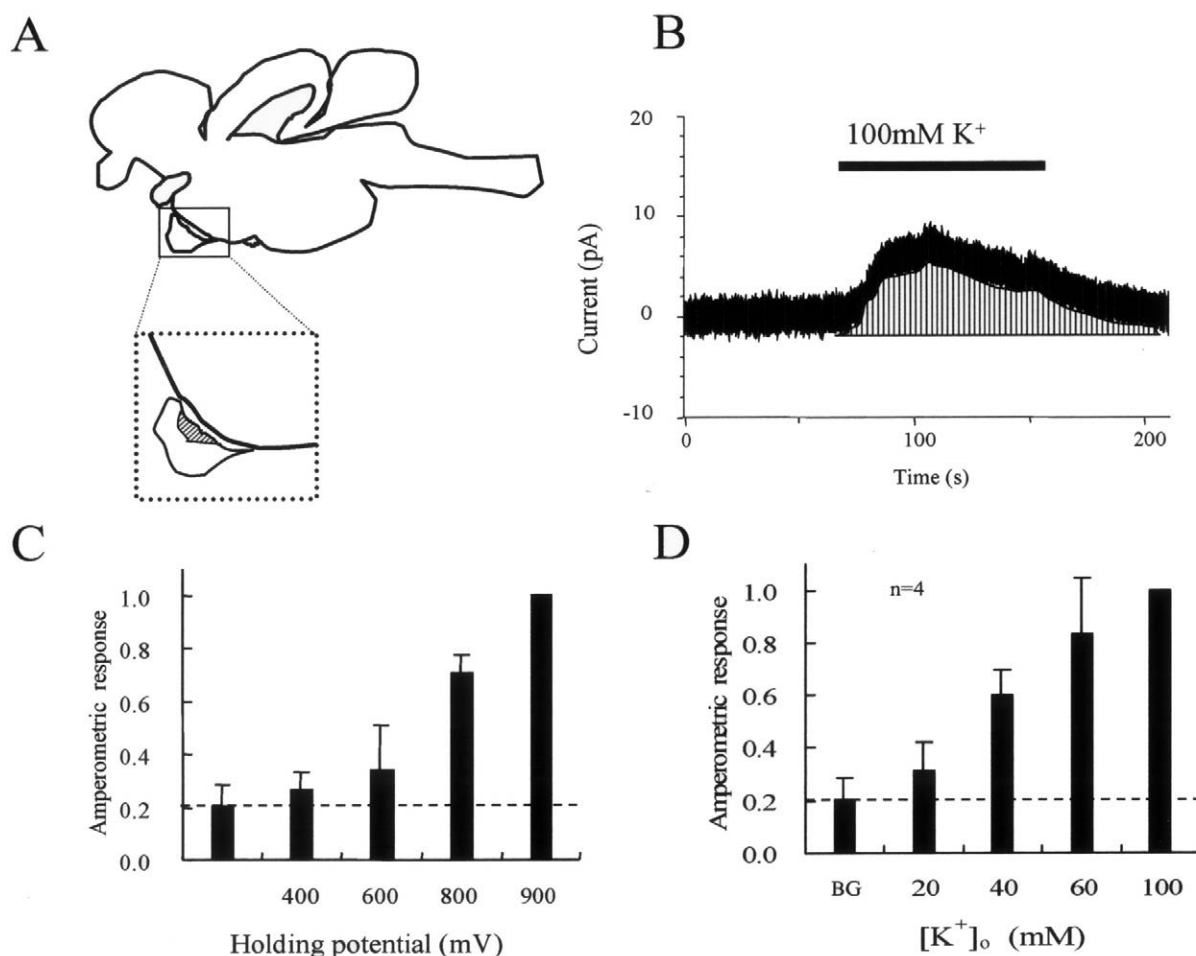


Fig. 6. Amperometric recording of release activities from the axon terminals in the pituitary. (A) The CFE was placed in the hatched area in the pituitary slice where the axon terminals from GnRH neurons are dense. (B) Amperometric recording ($V_h = 900$ mV) from the pituitary slice stimulated by high K^+ solution (100 mM). (C) Relationship between the V_h of the electrode and the amperometric current that was recorded from the pituitary slices stimulated by 100 mM K^+ . (D) $[K^+]_o$ -dependence of release activity of the pituitary slice ($V_h = 900$ mV). The data are normalized against the value for the maximal concentration of $[K^+]_o$ (100 mM). (Modified from Ishizaki and Oka, 2001.)

from the brain–pituitary slice preparation. V_h of CFE was held at 900 mV. A bulk current response was observed after bath application of a Ringer solution containing high K^+ concentration (100 mM) to the slice for 90 seconds. The time integral of this amperometric current, the charge transferred by oxidation, was calculated (Fig. 6B), and the release activity as measured by these values was dependent on the V_h of CFE (Fig. 6C). The amperometric response was also dependent on $[K^+]_o$, i.e., the degree of membrane depolarization (Fig. 6D). Because the amperometric response at the V_h lower than 600 mV was comparable to the background level, it was concluded that the contamination of the oxidation currents of catecholamines or serotonin was almost negligible. Since the voltage dependence of the amperometric current was comparable to that of the amperometric current in response to synthetic sGnRH, it is strongly suggested that the amperometric current in Fig. 6 is mainly attributed to the oxidation current of sGnRH peptide released from the axon terminals of POA-GnRH neurons in the pituitary. Although there are other axon terminals that secrete small peptide hormones such as isotocin and vasotocin in the teleost pituitary, it has been shown that the dorsal region of the pituitary where CFE was placed has the heaviest projection of GnRH-immunoreactive fibers but not isotocin-immunoreactive fibers in the dwarf gourami (Maejima et al., 1994). Furthermore, isotocin and vasotocin are considered to be very weakly electroactive, since they only contain one Tyr residue.

Thus, we succeeded in developing a new method that enables a real-time measurement of GnRH peptide release activity from the axon terminals in the pituitary in real-time using brain-pituitary slice preparations (Ishizaki and Oka, 2001). Although the currents recorded from the pituitary were not amperometric spikes but bulk currents, which may be attributed to the release from a large number of axon terminals, this technique will prove to be a powerful new tool for the study of GnRH release and may be applied to the real-time recording of release activity in other parts of the brain or in cell cultures that release GnRH. If the present method can be applied to the study of release activity of GnRH neurons in the brain, it may help us understand the functional significance of the multifunctional GnRH systems.

Working hypotheses

Fig. 7 illustrates our working hypothesis concerning the generation and modulation of pacemaker potentials of TN-GnRH cells. The TN-GnRH cells show regular pacemaker activities whose basic subthreshold rhythm is dependent on the interplay between the TTX-resistant persistent sodium current, $I_{Na(slow)}$, and a TEA-sensitive voltage-dependent potassium current, $I_{K(V)}$, persistent depolarizing drive and counteracting hyperpolarization, respectively. The store-operated Ca^{2+} current (SOC) may also partly contribute to the persistent inward current drive for the generation of pacemaker potentials. In addition, ω -conotoxin-sensitive N-type Ca^{2+} current(s) also seem to be involved in the suprathreshold (action potential) phase of the pacemaker potentials. The TN-GnRH neurons release GnRH not only from the varicosities and axon terminals but also from the somatodendritic areas. GnRH peptides released from the somatodendritic areas of the GnRH neurons facilitate the activities of their own (autocrine) and/or neighboring GnRH neurons (paracrine) and may cause synchronized positive feedback facilitation of multiple GnRH neurons. During this kind of modulation, the released GnRH peptide binds to the G-protein-coupled GnRH receptors in the cell membrane of TN-GnRH neurons and may function in the following manner.

(1) GnRH receptor activation facilitates Ca^{2+} release from intracellular Ca^{2+} store. The increased intracellular Ca^{2+} activates apamin-sensitive Ca^{2+} -dependent K^+ current(s) and decreases the frequency of pacemaker potentials transiently.

(2) The downstream signaling pathway somehow increases ω -conotoxin-sensitive N-type Ca^{2+} current(s) and hence the frequency of pacemaker potentials. Alternatively, it is also possible that Ca^{2+} influx through voltage-independent channels that are activated by depletion of Ca^{2+} stores (SOC) may be activated after (1) and are involved in the late phase increase of pacemaker frequencies (see van Goor et al., 1999). This kind of calcium influx may further be involved in the release of GnRH peptides (Fomina and Nowycky, 1999).

Similarly, changes in the physiological conditions of the fish that are triggered by environmental, pheromonal, hormonal factors, etc., probably act on

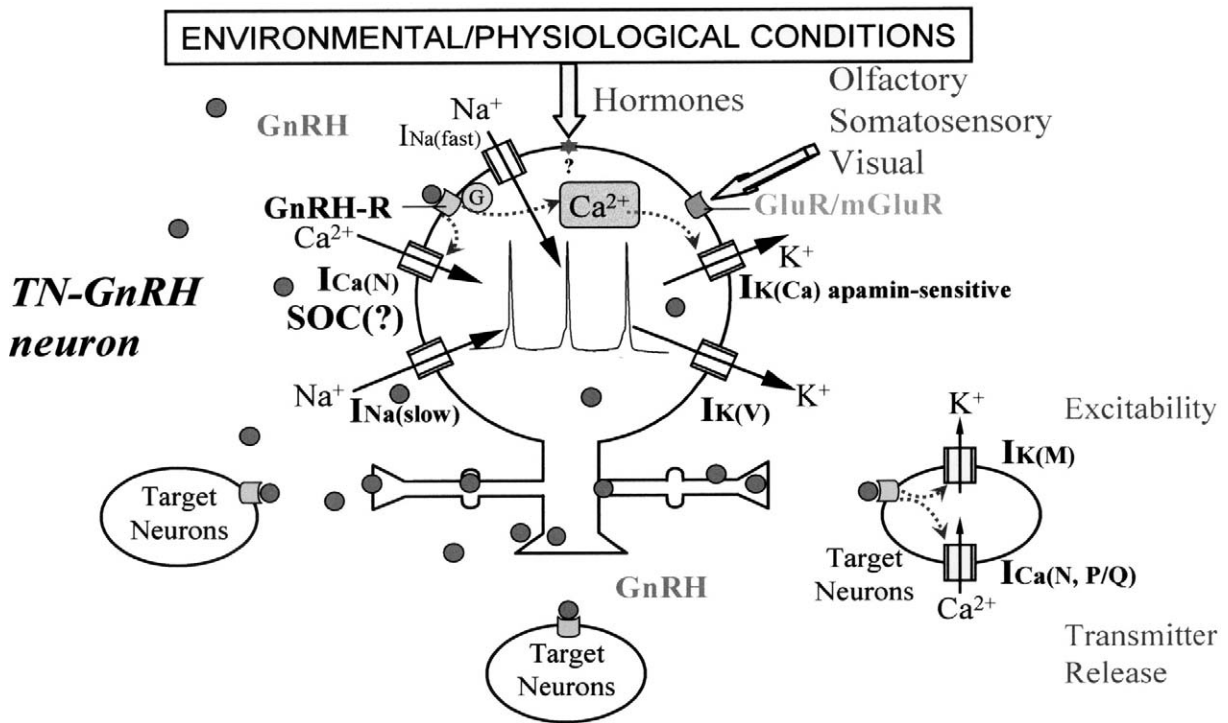


Fig. 7. Diagram illustrating the neuromodulator functions of TN-GnRH neurons, including some hypotheses. See text for details.

the GnRH neurons via hormones and/or neurotransmitters, and the properties of the ionic channels that underlie pacemaker activities may be modified by some kind of signal transduction mechanisms similar to the one described above. In this respect, it is interesting to note that some of the candidate GnRH neurons in ascidian neural ganglion (Tsutsui et al., 1998) may respond directly or indirectly to light stimuli (Tsutsui and Oka, 2000). In the dwarf gourami, Yamamoto and Ito (2000) reported on anatomical evidence for the somatosensory and visual inputs to the TN-GnRH neurons from the nucleus tegmento-terminalis in the midbrain tegmentum, and olfactory inputs from the olfactory bulb and the primary olfactory projection areas in the telencephalon. On the other hand, the TN-GnRH neurons are suggested to have various kinds of hormone receptors besides GnRH receptors; it has been reported in Tilapia (*Oreochromis niloticus*) that TN-GnRH neurons express receptors for thyroid hormone, testosterone, cortisol, etc., and these hormones are involved in the regulation of GnRH genes in TN-GnRH neurons (Soga et al., 1998; Parhar et al., 2000).

There are plenty of reports to show that GnRH peptide modulates several kinds of ion channels, e.g., potassium M current or N and P type I_{Ca} (see above). Since these currents are involved in the control of neuronal excitability and transmitter release, respectively, TN-GnRH cells may modulate the excitability of target neurons or presynaptic release of transmitters in wide brain regions simultaneously via extensive, multiple axonal branches. We have actually measured the exocytotic release of GnRH peptides from the brain slices containing the cell bodies and axons of TN-GnRH neurons by RIA and found that N-type Ca^{2+} currents and SOC, which we find are involved in the generation and modulation of pacemaker activities of GnRH neurons, are also involved in the control of GnRH release from the TN-GnRH slices. We hope that we can apply the real-time electrochemical measurement of GnRH release using a carbon fiber electrode (CFE) to measure the changes in GnRH release according to the changes in pacemaker frequencies of TN-GnRH neurons in near future. Finally, these cellular events may lead to a delicate control of the moti-

vational or arousal state in various aspects of the animal behavior.

As we have seen in this paper, there are multiple GnRH neuronal systems with different morphology, function, and molecular component, i.e., TN- and midbrain GnRH systems, which are considered to be neuromodulatory, and the hypophysiotropic POA GnRH system, which is the originally defined 'gonadotropin-releasing' system. In spite of these differences, the GnRH neuronal systems may have some basic characteristics in common as peptidergic neurons. Through a proper and deliberate synthesis of results obtained by using these diverse GnRH neuronal systems and multidisciplinary techniques, general principles concerning not only the GnRH neuronal system but also the peptidergic neuronal system as a whole will surely emerge. I believe that the teleost brain will greatly contribute to this exciting field of research.

Abbreviations

4AP	4-aminopyridine
ACh	acetyl choline
CFE	carbon fiber electrode
DCV	dense-cored vesicle
EM	electron microscopy
EPSP	excitatory postsynaptic potential
GFP	green fluorescent protein
GnRH	gonadotropin-releasing hormone
HRP	horse-radish peroxidase
nFLM	nucleus of the fasciculus medialis longitudinalis
POA	preoptic area
SOC	store-operated Ca^{2+} current
TN	terminal nerve
TTX	tetrodotoxin
Vh	holding potential

Acknowledgements

I would like to thank Dr. Yamamoto who contributed to the immunohistochemical and behavioral studies, Dr. Abe who contributed to the electrophysiological studies, and Ms. Ishizaki who contributed to the real-time CFE measurement of GnRH release. We would like to extend our gratitude to all of our colleagues and friends for help and discussion. This research

was supported by grant-in-aid for Fundamental Scientific Research from Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan to Y. Oka (#10554050 and #12440237).

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A role for non-neuronal cells in synchronization of intracellular calcium oscillations in primate LHRH neurons

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Introduction

Until the discovery of the LHRH molecule in 1971 by Schally and his collaborators (Schally et al., 1971), the hypothalamus remained a black box in the hypothalamo–pituitary–gonadal axis. In the last three decades, a quantum leap in our understanding on the hypothalamic control of reproductive function has been made. In primates, approximately 2000 LHRH neurons originate from the olfactory placode during the early embryonic stages and settle down in the septum–preoptic regions and basal hypothalamus (Anthony et al., 1984; Ronnekleiv and Resko, 1990; Quanbeck et al., 1997). Of these 2000 LHRH neurons, only a subset distributed in the medial basal hypothalamus is necessary and sufficient for sustaining reproductive function, since only lesion, but not complete deafferentation, of the medial basal hypothalamus interferes with ovulatory cycles (Krey et al., 1975; Plant et al., 1978).

LHRH is released in a pulsatile manner into the portal circulation (Carmel et al., 1976; Clarke and Cummins, 1982). An increase in the frequency and amplitude of LHRH pulses are essential for the onset of puberty (Terasawa and Fernandez, 2001) and the maintenance of regular LHRH pulsatility is important for normal reproductive function (Knobil, 1980,

1988). A study using multiunit activity recording from the medial basal hypothalamus indicates that pulsatile LHRH release is a consequence of synchronized activity of individual LHRH neurons. An increase in single unit spike components extracted from the multiunit ‘LHRH pulse-generator’ activity (Wilson et al., 1984) by cluster analysis occurs as a simultaneous increase in the firing rate of many individual hypothalamic neurons rather than the recruitment of new bursting neurons (Cardenas et al., 1993).

The mechanism underlying LHRH synchronization is currently unknown. This is because, unlike oxytocin and vasopressin neurons, LHRH neurons do not form a nucleus in the hypothalamus (Silverman, 1988) and there is little evidence for physical contact between LHRH neurons, such as synapses or syncytium formation, that would allow the synchronization of LHRH neurons (Witkin, 1999). It is, therefore, hypothesized that non-neuronal elements, such as glia, may coordinate activity among LHRH neurons (Terasawa, 1995, 2001). Here we report evidence that non-neuronal cells in cultures containing LHRH neurons derived from the embryonic olfactory placode participate in the synchronization of LHRH neurons.

LHRH release from cultured neurons

Previously, we have shown that cultures from the olfactory placode and terminal nerve region obtained from monkey embryos at embryonic day (E) 35–37 contain LHRH neurons, which are easily identi-

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able because of their unique appearance (Terasawa et al., 1993). These cultures also contain non-neuronal cells, such as epithelial cells and fibroblasts, but there are few non-LHRH neurons and no glia (Terasawa et al., 1993). Cultured LHRH neurons release the decapeptide in a pulsatile manner at intervals of approximately an hour (Terasawa et al., 1999a), similar to that observed *in vivo* (Dierschke et al., 1970; Knobil, 1980; Gearing and Terasawa, 1988) and LHRH release is dependent on the presence of extracellular calcium, $[Ca^{2+}]_e$ (Terasawa et al., 1999a). LHRH release is also induced by depolarization stimuli, such as challenge with high K^+ and the Na^+ channel opener, veratridine (Terasawa et al., 1999a). Ca^{2+} enters LHRH neurons through voltage-sensitive L-type Ca^{2+} channels and stimulates LHRH release (Terasawa et al., 1999a). Pulsatile release of LHRH was also reported in placode cultures from sheep and rats (Duittoz and Batailler, 2000; Funabashi et al., 2001) as well as in GT1 cells (Krsmanovic et al., 1992; Martinez de la Escalera et al., 1992; Wetsel et al., 1992). Interestingly, the interval of pulsatile LHRH release *in vitro* is species specific, as reported *in vivo*, i.e., approximately 60 min in sheep (Duittoz and Batailler, 2000) and 30 min in rats (Funabashi et al., 2001) and GT1 cells, which are of mouse origin (Krsmanovic et al., 1992; Martinez de la Escalera et al., 1992; Wetsel et al., 1992).

Synchronization of intracellular Ca^{2+} oscillations in LHRH neurons and non-neuronal cells

Individual LHRH neurons exhibit periodic increases in intracellular Ca^{2+} , $[Ca^{2+}]_i$, concentrations (Charles and Hales, 1995; Charles et al., 1996; Terasawa et al., 1999b; Moore and Wray, 2000; Nunez et al., 2000) and an $[Ca^{2+}]_i$ increase is preceded by increases in electrical firing activity in GT1 cells (Costantin and Charles, 1999). Moreover, $[Ca^{2+}]_i$ oscillations in cultured primate LHRH neurons synchronize at intervals of approximately 60 min (Terasawa et al., 1999b). Similar synchronization of $[Ca^{2+}]_i$ oscillations in GT1 cells also has been reported with much shorter intervals (Charles et al., 1996). Electrical activities recorded by a low impedance multiple plate electrode recording system reveal that many GT1 neurons also exhibit synchronous neural activities (Funabashi et al., 2001;

Nunemaker et al., 2001). However, since in the hypothalamus the LHRH neurosecretory system that releases LHRH in a pulsatile manner is intermingled with other neurons and glia, unlike GT1 cells cultures, the non-LHRH cells in addition to LHRH neurons in our cultures allow us to address the question of whether the activity of only LHRH neurons is synchronized or whether non-LHRH cells are involved in this phenomenon.

A previous study (Terasawa et al., 1999b) indicated that olfactory placode cultures containing LHRH neurons exhibit periodic synchronization of $[Ca^{2+}]_i$ oscillations (Fig. 1). A recent study (Richter et al., 2002) further indicated that both LHRH neurons and non-neuronal cells in the same cultures exhibited spontaneous $[Ca^{2+}]_i$ oscillations with similar interpeak intervals (IPI). Moreover, $[Ca^{2+}]_i$ peaks in individual LHRH neurons and non-neuronal cells were periodically synchronized across the cell population (Fig. 1; Richter et al., 2002). In fact, $[Ca^{2+}]_i$ peaks in many cells often occurred within a narrow window of time (~ 20 sec, Terasawa et al., 1999b; Richter et al., 2002). When synchronization occurred in the majority of the cell population (80–100% of cells), the interval of synchronization was ~ 60 min (Fig. 1; Richter et al., 2002), which is similar to the interval of LHRH release *in vitro* (Terasawa et al., 1999a) and *in vivo* (Knobil, 1988). The amplitude of highly synchronized $[Ca^{2+}]_i$ peaks (80–100% of cells in a population) was significantly ($p < 0.05$) larger than that of unsynchronized peaks (Richter et al., 2002), and the IPI immediately after the highly synchronized $[Ca^{2+}]_i$ peaks among LHRH neurons was greater than for other $[Ca^{2+}]_i$ peaks (Fig. 2). The IPI immediately following highly synchronized $[Ca^{2+}]_i$ pulses among non-neuronal cells did not differ from non-synchronized peaks (Richter et al., 2002). These observations suggest that synchronization of $[Ca^{2+}]_i$ oscillations is an organized cell activity through which the cells in the network communicate with each other for a certain function, such as LHRH neurosecretion.

Synchronization of oscillations in $[Ca^{2+}]_i$ appeared as intercellular Ca^{2+} waves that spread across fields containing both LHRH neurons and non-neuronal cells. However, propagation of $[Ca^{2+}]_i$ oscillations in LHRH neurons differs from that in non-LHRH cells: the average speed at which Ca^{2+} waves

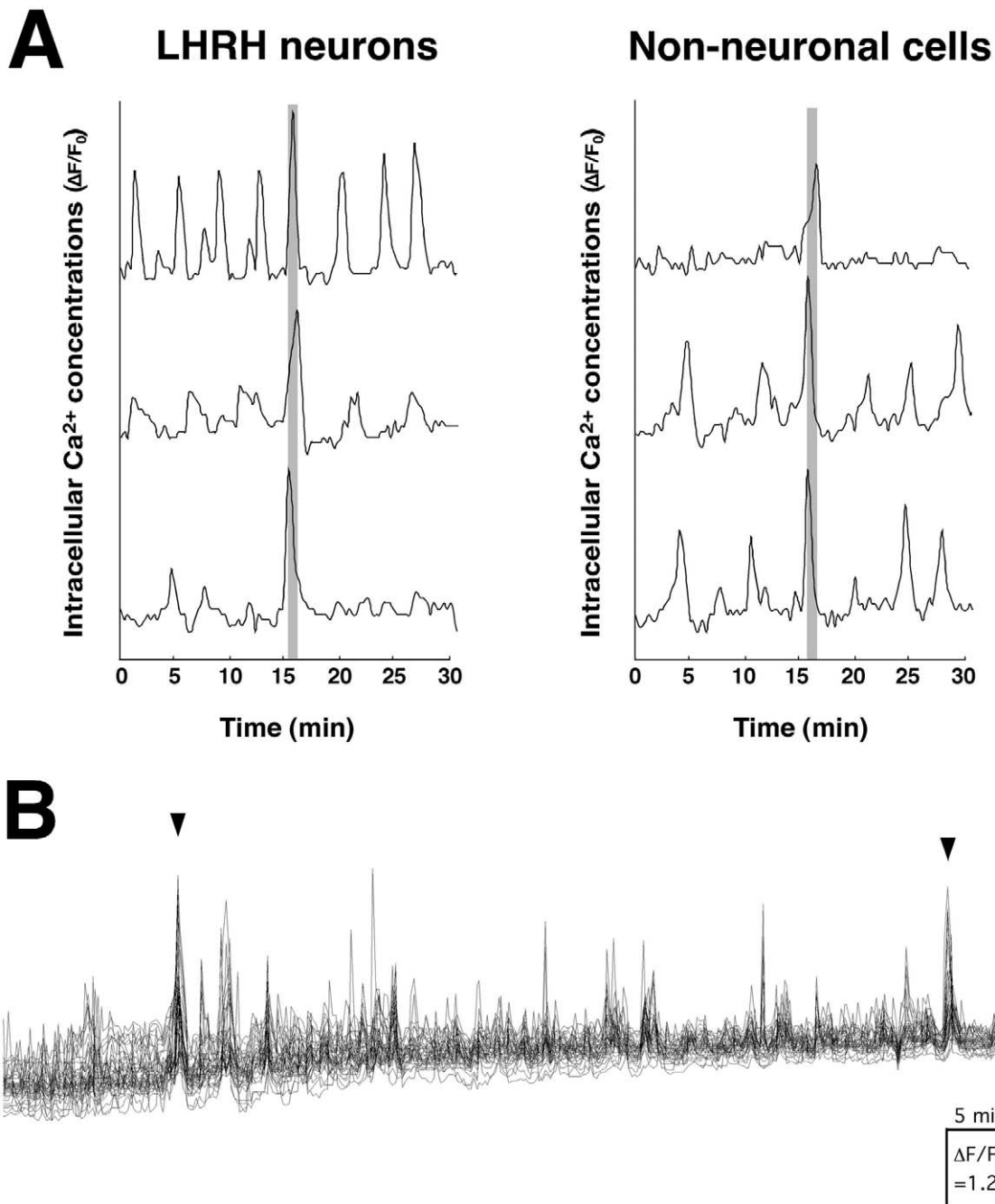


Fig. 1. (A) Examples of $[\text{Ca}^{2+}]_i$ oscillations that are periodically synchronized among LHRH neurons (left) and non-neuronal cells (right), from the same olfactory placode culture. The traces represent changes in $[\text{Ca}^{2+}]_i$ concentrations in 3 LHRH neurons and 3 non-neuronal cells obtained simultaneously by ratiometric measurements using fura2-AM. The shaded regions indicate narrow windows of time within which each of the cells exhibited a $[\text{Ca}^{2+}]_i$ peak. (B) An example of synchronization of $[\text{Ca}^{2+}]_i$ peaks occurring at an interval of ~ 60 min. Arrowheads indicate synchronization of $[\text{Ca}^{2+}]_i$ peaks among $>80\%$ of 72 individual cells ($n = 34$ LHRH neurons, 38 non-neuronal cells) in the culture. (Based on Richter et al., 2002.)

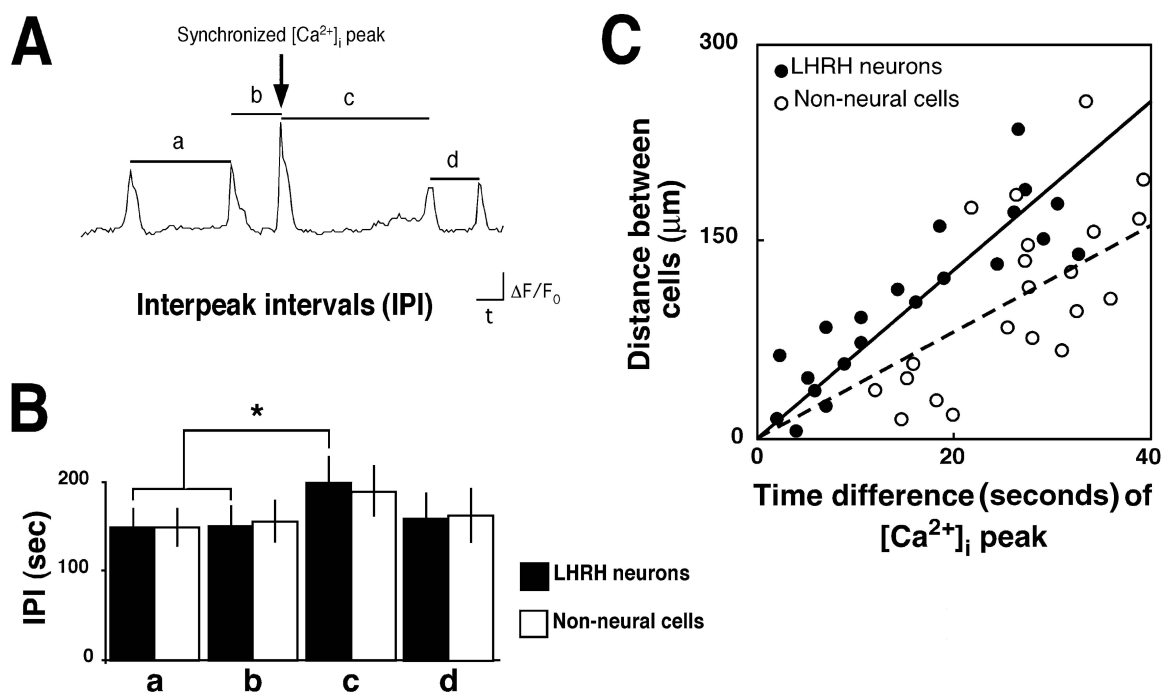


Fig. 2. The interpeak interval (IPI) after highly synchronized $[Ca^{2+}]_i$ peaks ($>80\%$ of cells synchronized) was longer than other IPIs. (A) The IPI was calculated for the two peaks in $[Ca^{2+}]_i$ that immediately preceded (a, b) or immediately followed (c, d) a peak that was highly synchronized among cells (arrow). The tracing is from a representative individual cell. (B) Mean (\pm SE) IPI for LHRH neurons (solid bars) and non-neuronal cells (open bars) for the IPIs (a–d) illustrated in A. Data represent synchronizations from 7 different cultures ($n = 628$ cells). *, $p < 0.05$, ANOVA with Fisher's PLSD post hoc test. (C) Ca^{2+} waves that were associated with synchronized oscillations in $[Ca^{2+}]_i$ spread more rapidly in LHRH neurons than in non-neuronal cells. Data represent the distance between cells vs. the time at which a peak in $[Ca^{2+}]_i$ occurred during synchronization. To generate this plot, we grouped data in bins according to the mean distance between cells ($0\text{--}10\text{ }\mu m$, $10\text{--}20\text{ }\mu m$, . . . , $>200\text{ }\mu m$; $n = 21$ bins). For LHRH neurons (closed symbols, solid line), $Y = 7.05X$, $r^2 = 0.67$, $p < 0.001$. For non-neuronal cells (open symbols, broken line), $Y = 4.48X$, $r^2 = 0.41$, $p = 0.001$. The slope of the regression for LHRH neurons was significantly ($p < 0.01$, Student's t -test, $n = 21$) greater than for non-neuronal cells, indicating that Ca^{2+} waves were propagated more rapidly in LHRH neurons than in non-neuronal cells ($17.6\text{ }\mu m/s$ in LHRH neurons vs. $11.1\text{ }\mu m/s$ in non-neuronal cells). (Based on Richter et al., 2002.)

propagated among LHRH neurons ($17.6\text{ }\mu m/s$) was significantly ($P < 0.01$) faster than that among non-neuronal cells ($11.1\text{ }\mu m/s$, Fig. 2; Richter et al., 2002). Similar propagation of intercellular signals in GT1 cells has been also reported: An elevation of $[Ca^{2+}]_i$ originating in a cell is propagated to adjacent cells (Charles et al., 1996) and neuronal excitation monitored by a voltage-sensitive dye periodically spreads across adjacent cells (Hiruma et al., 1997). Moreover, highly synchronized $[Ca^{2+}]_i$ oscillations and intercellular Ca^{2+} waves occur only in cultures that contain LHRH neurons and are absent from cultures that do not contain LHRH neurons (Richter et al., 2002). Although the source initiating the synchronized $[Ca^{2+}]_i$ oscillations in our cultures is un-

clear at this time, this observation clearly suggests that a LHRH neuron or group of LHRH neurons is responsible for initiating synchronization.

Ca^{2+} waves occur in many different types of cell, including glia, neurons, epithelial cells, endothelial cells, hepatocytes, and pancreatic β -cells (Cornell-Bell et al., 1990; Sanderson et al., 1990; Boitano et al., 1992; Nathanson et al., 1995; Charles et al., 1996; Cao et al., 1997; Newman and Zahs, 1997; Harris-White et al., 1998; Guthrie et al., 1999; Cotrina et al., 2000). Because neuronal activity can induce Ca^{2+} waves in glia (Dani et al., 1992), and glial Ca^{2+} waves induce Ca^{2+} transients and electrical activity in neurons (Nedergaard, 1994; Hassinger et al., 1996; Newman and Zahs, 1997), the concept has been developed

that the calcium wave is a mode of intercellular communication not only among glial cells or neurons, but also between neurons and glia. It is possible, therefore, that in our cultures non-neuronal cells play a role similar to that of glial cells in the brain and participate in synchronizing LHRH neural activity.

Cellular elements participating in the synchronization of LHRH neural activity

It has been reported that perikarya of LHRH neurons are covered by glial sheathes (Witkin et al., 1991) and LHRH neuroterminals are intimately associated with glial end feet (King and Rubin, 1994). Previously, we hypothesized that glia play an important role in LHRH pulsatility (Terasawa, 1995). This hypothesis was based upon the facts that (1) pulsatile LHRH release occurs within the rat stalk–median eminence (Maeda et al., 1995; Purnelle et al., 1997), where an abundance of neuroterminals and glial cells are present, but not perikarya of LHRH neurons (Silverman, 1988), (2) neuropeptide Y (NPY) and norepinephrine (NE) are also released in a pulsatile manner that is coupled with LHRH pulses (Terasawa et al., 1988; Woller et al., 1992), but LHRH neurons receive little direct synaptic input from NPY and NE neurons (Thind et al., 1993), suggesting a role for glial communication among those neuronal systems, (3) glia play a critical role in release of oxytocin and vasopressin (Hatton, 1985), and (4) glia appear to play a role in a part of the signaling pathway of neurons (Parpura et al., 1994; Araque et al., 1999; Haydon, 2000, 2001; Ullian et al., 2001).

In our cultures, synchronization of $[Ca^{2+}]_i$ oscillations among cells is propagated as an intercellular Ca^{2+} wave across fields of cells containing both LHRH neurons and non-neuronal cells. A similar synchronization pattern of electrical activity in simulated networks of coupled cells, in which sparse, random activation of individual cells occurs spontaneously to produce intercellular activity waves, has been reported (Lewis and Rinzel, 2000). Since these authors show that in theoretical model systems the development of intercellular waves requires some form of coupling among neighboring cells (Lewis and Rinzel, 2000), the propagation of Ca^{2+} waves through fields including LHRH neurons and non-neuronal cells in our cultures is interpreted to mean

that LHRH neurons are functionally coupled to non-neuronal cells. Similar integrated signaling between neurons and non-neuronal cells has also been shown in cultures of rat forebrain tissue, where astrocytes are able to transmit Ca^{2+} signals to neurons (Nedergaard, 1994). Thus, non-neuronal cells, such as glia, might be a crucial component of the LHRH neurosecretory system in vivo, providing an indirect coupling mechanism to facilitate the synchronization of isolated LHRH neurons.

Possible chemical mechanisms mediating interneuronal signals

As candidate intercellular messengers, nitric oxide (NO, Snyder, 1992; Schuman and Madison, 1994), ATP (Osipchuk and Cahalan, 1992; Newman and Zahs, 1997; Guthrie et al., 1999; Cotrina et al., 2000), inositol 1,4,5-trisphosphate (IP_3 , Charles et al., 1996), glutamate (Parpura et al., 1994), as well as cations, such as K^+ and Ca^{2+} (Nedergaard, 1994), have been described.

NO, a free radical gas produced during the conversion of arginine to citrulline in the presence of NO synthase, is involved in control of LHRH pulsatility (Brann and Mahesh, 1997; Kawakami et al., 1998). NO induces LHRH release in GT-1 cells (Sortino et al., 1994) and NO synthase mRNA is present in GT1 cells (Mahachoklertwuttana et al., 1994). An in vivo study in our laboratory has also shown that NO can induce both NPY and LHRH release from the stalk–median eminence: Using a push–pull perfusion method in the rhesus monkey, infusion of L-arginine, the precursor for NO production, stimulated NPY and LHRH release, while D-arginine (as a control) failed to cause any increase in release (Terasawa and Nyberg, 1997). Although we found the presence of nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase, which is a subtype of NO synthase (Wang et al., 1997), in fibers located in the infundibular nucleus and median eminence region, and in perikarya located in the perimammillary region of the hypothalamus (phenotypes of fibers and perikarya yet to be clarified) in rhesus monkeys (unpublished observation), the physiological role of these cells is yet to be determined.

Intercellular diffusion of various small molecules such as ions, second messengers and metabolites be-

tween cells can occur through transmembrane channels, namely gap junctions, without direct physical contact of the cells. Gap junctions are particularly important for intercellular communication, because they are widespread and permit rapid intercellular transit. The presence of dye-coupling between GT1 cells has been reported (Liposits et al., 1991; Wetsel et al., 1992), and connexin 26, a protein associated with gap junctions, was found in GT1 cells (Krsmanovic et al., 1992; Matesic et al., 1993; Hu et al., 1999). LHRH neurons in the hypothalamus (Hosny and Jennes, 1998) and in our cultures (Terasawa, unpublished observation) are immunopositive to connexin 32, another protein associated with gap junctions. Moreover, in our cultures Lucifer yellow injected into single non-neuronal cells diffuses into neighboring non-neuronal cells as well as to LHRH neurons (Richter, Wolfgang, and Terasawa, unpublished data). Finally, blocking gap junctions with octanol or carbenoxolone abolishes intercellular Ca^{2+} waves and synchronous electrical activity in GT1 cells (Charles et al., 1996; Funabashi et al., 2001).

Recent preliminary studies suggest that the synchronization of $[\text{Ca}^{2+}]_i$ oscillations in our cultures may be mediated by extracellular adenosine triphosphate (ATP) diffusing through gap junctions and/or purinergic receptors, P2X subtypes: (1) ATP, but neither ADP nor AMP, induced $[\text{Ca}^{2+}]_i$ increases with a dose-responsive manner, and (2) ATP resulted in a significant increase in LHRH release. Nonetheless, the observation that the speed of Ca^{2+} waves is faster among neurons than non-neuronal cells (Fig. 2; Richter et al., 2002) suggests that neurons are equipped with additional mechanisms for intercellular communication, such as synaptic neurotransmission. Although we have yet to confirm the presence of synapses between LHRH neurons in our cultures, relatively immature synapses between GT1 neurons have been reported (Liposits et al., 1991).

It has been shown in GT1 cells that depolarization always precedes $[\text{Ca}^{2+}]_i$ increases (Costantin and Charles, 1999). This important observation suggests that Ca^{2+} waves (i.e., synchronization) are propagated by initial depolarization and subsequent chemical diffusion of substances, such as NO, ATP, IP_3 , K^+ , and Ca^{2+} , as well as synaptic transmission. Further studies are needed in this regard.

Synchronization of $[\text{Ca}^{2+}]_i$ oscillations and LHRH neurosecretion

We have reported that highly synchronized $[\text{Ca}^{2+}]_i$ oscillations occur at a frequency similar to LHRH release in vitro (Terasawa et al., 1999b) and in vivo (Knobil, 1988). A recent study suggests that synchronization of $[\text{Ca}^{2+}]_i$ oscillations in GT1 neurons is associated with increases in membrane area, which is indicative of neurosecretion (Vazquez-Martinez et al., 2001). Therefore, we hypothesize that highly synchronized oscillations in $[\text{Ca}^{2+}]_i$ could provide a stimulus for LHRH neurosecretion. Since neurosecretion requires an increase in $[\text{Ca}^{2+}]_i$, the increase in $[\text{Ca}^{2+}]_i$ that occurs during a highly synchronous event could produce a suprathreshold elevation of $[\text{Ca}^{2+}]_i$ that triggers neurosecretion. The finding in a previous study that the amplitude of $[\text{Ca}^{2+}]_i$ peaks of synchronized oscillations was significantly larger than non-synchronized peaks supports this hypothesis. In fact, the larger peak amplitude might reflect an increase in $[\text{Ca}^{2+}]_i$ that is not only sufficient (i.e. a suprathreshold stimulus) to stimulate Ca^{2+} -dependent neurosecretion, but would also cause concomitant increases in $[\text{Ca}^{2+}]_i$ in neighboring cells, thereby transmitting a neurosecretion-inducing stimulus to other neurons. In addition, the observation that the IPI following a highly synchronous $[\text{Ca}^{2+}]_i$ peak is greater than IPIs at other times might be due to the additional time required for ion pumps on external and internal membranes to redistribute intracellular Ca^{2+} that has been released into the cytoplasm from endoplasmic reticulum and mitochondrial stores following a large amplitude, highly synchronized $[\text{Ca}^{2+}]_i$ increase. The simultaneous measurement of LHRH neurosecretion and $[\text{Ca}^{2+}]_i$ oscillations in single cells is needed to test the aforementioned hypothesis.

Conclusion

The study of the mechanism of LHRH pulse generation is important, since abnormalities in pulsatile LHRH release are associated with reproductive disorders in humans (Crowley et al., 1985; Spratt et al., 1987). Primate olfactory placode cultures which contain LHRH neurons and other cells exhibit hourly rhythms of LHRH release and synchronization of

$[Ca^{2+}]_i$ oscillations. Synchronization of $[Ca^{2+}]_i$ oscillations occurs among LHRH neurons and non-LHRH cells, suggesting that LHRH neurons form a functional network with non-neuronal cells for pulsatile LHRH release. Moreover, synchronization of $[Ca^{2+}]_i$ oscillations in non-LHRH cells occurs only when LHRH neurons are present in cultures, indicating that LHRH neurons are responsible for the initiation of synchronization. Translating these observations into events in the hypothalamus, we speculate that a similar mechanism may exist between LHRH neurons and glia in the medial basal hypothalamus for LHRH release in vivo. Much work, however, remains including elucidating: (1) the mechanism generating periodic $[Ca^{2+}]_i$ oscillations, (2) the mechanism determining the timing of $[Ca^{2+}]_i$ synchronization in LHRH neurons, (3) the molecule(s) responsible for intercellular communication among LHRH neurons and non-LHRH cells including glia, and (4) the intracellular mechanism that translates LHRH neuronal activity into neurosecretion. It is our hope that the answers to these questions will be forthcoming in the near future.

Acknowledgements

A portion of this review was based on studies supported by NIH grants HD15433, HD11533 and RR00167 (publication number 42-004 from the Wisconsin Regional Primate Research Center).

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GnRH in the regulation of female rat sexual behavior

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Introduction

The potency of gonadotropin-releasing hormone (GnRH) to enhance the lordosis reflex, principal estrogen-sensitive component of female rat sexual behavior, was demonstrated soon after the isolation of the mammalian decapeptide and the determination of its amino-acid sequence (Moss and McCann, 1973; Pfaff, 1973). It has been hypothesized that GnRH may play a role in synchronizing the timing of ovulation and behavioral receptivity. GnRH prolongs estrogen-induced receptivity in the ovariectomized ewe (Caraty et al., 2002). At the same time, the existence of different subsets of mammalian GnRH neurons have been suggested to be involved in the neuroendocrine and behavioral regulation in the mouse (Skynner et al., 1999b; Rajendren, 2001). Besides, more than a dozen isoforms of GnRH, which are conserved by 50–90% in their amino-acid sequence, are known across species (Sherwood et al., 1993). In the brain of many vertebrates, two or three isoforms of GnRH are expressed along with unique receptors (King and Millar, 1995). In the brain of the mouse, rat and primate, two isoforms of GnRH (GnRH-II and III) have been identified in addition to the original mammalian decapeptide, which is now sometimes referred to as GnRH-I (Lescheid et al., 1997; Chen et al., 1998; White et al., 1998). GnRH-II or [His⁵,Trp⁷,Tyr⁸] GnRH, isolated from

chicken brain (King and Millar, 1984; Miyamoto et al., 1984), is expressed in almost all vertebrate classes. Chromatographic elution pattern and antigenicity suggest that GnRH-III is similar to salmon GnRH (sGnRH, [Trp⁷,Leu⁸]GnRH), which is ubiquitous in the terminal nerve ganglia of many teleosts (Parhar et al., 2000). In addition to these natural isoforms of GnRH, many GnRH analogs have been synthesized in attempts to exploit them as potent drugs to control fertility.

Expression of GnRH and its message is enhanced in the preoptic and limbic structures in the female rat in estrus (Malik et al., 1991). Estrogen enhances the expression of both GnRH receptor message and molecule (Jennes and Conn, 1994). Although the lack of nuclear estrogen receptor α (ER α), which is critically involved in the induction of the lordosis reflex (Ogawa et al., 1998), rules out possible direct regulation of the expression of GnRH by estrogen (Watson et al., 1992; Leng, 1999), changes in brain content of GnRH during the estrous cycle (Malik et al., 1991), persistent estrous females (Ma et al., 1990) or those changes that follow estrogen treatment of the ovariectomized females (Malik et al., 1991; Petersen et al., 1995) suggest transsynaptic or other non-genomic mechanism for estrogen to regulate GnRH turnover. Estrogen-dependent regulation of GnRH may also depend on ER β , which is colocalized in certain GnRH neurons in the rat preoptic area (Kallo et al., 2001). Thus, the theory that GnRH mediates and synchronizes neuroendocrine and behavioral events may be valid in principle, albeit its mechanism of action appears to be far more complicated than it was thought initially.

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Neural actions of GnRH

Neural actions of GnRH underlie changes in behavioral expression induced by this peptide. GnRH is present in dense core vesicles of presynaptic terminals in the diagonal band and preoptic area, suggesting that the peptide probably acts as a transmitter in these structures (Chen et al., 1990). GnRH modulates the cholinergic transmission in frog neuromuscular junction (Akasu et al., 1983). Behavioral and neural effects of GnRH have been demonstrated by a variety of methods in the preoptic, medial basal hypothalamic and limbic structures. GnRH bindings were found in high concentrations in the dorsal hippocampus, amygdala, septum, and subiculum and in low amounts in the hypothalamus. Generally, GnRH molecule and receptor distribution is in good correlation in these structures (Jennes and Conn, 1994).

We have shown that microiontophoresis of GnRH causes excitation in many neurons in the female rat hypothalamus and preoptic area (Kawakami and Sakuma, 1974). Neurons in brain slices taken from the female rat ventromedial nucleus and preoptic area responded to GnRH in perfusate *in vitro* experiments (Kow and Pfaff, 1988). A direct evidence for a modulatory role of GnRH in the synaptic transmission has been provided by intracellular recordings from postganglionic neurons in the bullfrog sympathetic ganglia, in which a GnRH-like peptide functions as the transmitter for the exceptionally slow excitatory postsynaptic potential that lasts several minutes (Kuffler, 1980).

A brief overview on the effects of GnRH on neuronal activities in discrete brain structures, that have been associated with the regulation of female rat sexual behavior, follows.

Preoptic area

In the rat and in many vertebrates, a major population of GnRH neurons has been identified in the diagonal band–medial septum–medial preoptic continuum. GnRH neurons in these rostral structures have been emphasized not only in the neuroendocrine regulation of the anterior pituitary but also experiments in the ferrets or hypogonadal mice associate these neurons with behavioral regulation. This view is supported by observations that approximately

50% of the GnRH neurons project to the median eminence and are capable of neuroendocrine control, while others project elsewhere and are apparently involved in peptidergic neurotransmission (Jennes and Stumpf, 1986; Silverman et al., 1987). The fact that at the time of the preovulatory gonadotropin surge, Fos expression (Lee et al., 1990) or GnRH gene activation (Porkka-Heiskanen et al., 1994) occurs in a limited number of GnRH neurons also collaborates this interpretation.

Hypogonadal female mice, that are genetically deficient in GnRH neurons, show no sexual activity and are infertile. Implants of fetal brain grafts that contain GnRH neurons in the third ventricle induce vaginal opening and persistent vaginal estrus, ovarian, and uterine development and increased gonadotropin secretion. When these females are mated with normal males, they show comparable levels of lordosis as seen in normal female mice in estrus (Gibson et al., 1987). Immunocytochemistry revealed innervation of the median eminence, but not the midbrain central gray by axons originating from preoptic implants (Saitoh et al., 1992). Further elaboration is needed on the role played by GnRH and analogs in the midbrain, in light of the detection of GnRH-II in this region (Rissman et al., 1995).

Midbrain central gray

In the female rat, GnRH-immunoreactive nerve fibers descend in the midbrain central gray and continue as far caudal as the ponto-mesencephalic junction (Liposits and Setalo, 1980; Buma, 1989). Autoradiography and radioligand assays revealed substantial binding of GnRH in the central gray (Badr and Pelletier, 1987; Jennes et al., 1988). The numbers of central gray neurons that are excited by the microiontophoresis of GnRH increase following estrogen treatment in the ovariectomized female rat (Chan et al., 1985; Schiess et al., 1987). Taken together with morphological demonstration of estrogen-receptor positive neurons in the central gray (Murphy and Hoffman, 2001), the results of the microiontophoresis study suggest an estrogen-dependent regulation of GnRH receptors in the central gray. The cellular mechanisms for this enhanced sensitivity are unclear, but a distinct possibility is that GnRH receptor function may be enhanced by

either increase in number or coupling to GnRH, as has been suggested for pituitary gonadotrophs (Bauer-Dantoin et al., 1995; Ortmann and Diedrich, 1999). Indirect modulation of the neuronal activity cannot be ruled out as a route for GnRH action. The excitatory GnRH effect on central gray neurons in perfused midbrain slices *in vitro* has been attributed to modulation of GABAergic or adrenergic transmission (Ogawa et al., 1992). In any case, evidence abounds that GnRH promotes the lordosis reflex via its excitatory action on neurons in the midbrain central gray (Sakuma and Pfaff, 1980; Riskind and Moss, 1983a,b; Sakuma and Pfaff, 1983; Sirinathsinghji, 1984), similar to electrical stimulation of this structure (Sakuma and Pfaff, 1979).

The central gray is not a single entity and contains neural components that inhibit the lordosis reflex. Contrary to the dorsal subdivision of the central gray (Sakuma and Pfaff, 1979), electrical stimulation of the ventral central gray disrupts lordosis (Arendash and Gorski, 1983). The effect is probably due to the activation of axons of passage that originate in the ventral tegmental area (Sakamoto et al., 1993) and it is unlikely that GnRH interacts with these axons. Functional dichotomy between the ventral and dorsal subdivisions is also known for pain control (Cannon et al., 1982) or autonomic regulation (Lovick, 1991).

The limbic structures

GnRH receptors are densely expressed in the medial amygdala, hippocampus and septum (Badr and Pelletier, 1987; Jennes et al., 1988). Microiontophoresis of GnRH causes excitatory response in a subset of individual neurons in the medial amygdala and the effect is augmented by estrogen (Dudley et al., 1990). In some cells, electrical stimulation of the medial septum similarly excites their activity. Although estrogen enhances the sensitivity of individual neurons to GnRH, it decreases the percentage of the neurons that are excited by septal stimulation (Dudley et al., 1990). On the other hand, bilateral lesion of the medial amygdala diminished the lordosis reflex, simultaneously with a reduction of septal preoptic GnRH neurons that exhibit Fos immunoreactivity (Pfaus et al., 1996). Taken together, these findings suggest that GnRHergic neurons, that employ GnRH as an excitatory neurotransmitter or neuromodulator,

reside in the septal–preoptic continuum and project to the medial amygdala. They may constitute a neural substrate for olfactory enhancement of a facilitatory system of the lordosis reflex (Rajendren and Moss, 1993).

Hippocampal pyramidal cells, particularly those in the CA1 and CA3 regions, express GnRH receptors (Jennes et al., 1988). Intracellular recordings from CA1 neurons revealed that GnRH decapeptide and its behaviorally active fragment Ac-LHRH^{5–10} induce long-lasting depolarization (Chen et al., 1993). However, septo-preoptic GnRH neurons do not project to the hippocampus (Dudley et al., 1992). A more stable C-fragment catabolites derived from the hydroxylated GnRH, which also possesses behavioral actions, has been proposed as a possible natural ligand for hippocampal GnRH receptors (Gautron et al., 1993).

Ventral tegmental area

The presence of fibers with GnRH immunoreactivity (Siverman and Krey, 1978; Merchenthaler et al., 1984) and GnRH agonist binding sites (Jennes et al., 1988) in the ventral tegmental area suggest a role for GnRH in the neural transmission in this structure. Indeed, microinfusion of GnRH into the ventral tegmental area enhances the lordosis reflex (Sirinathsinghji et al., 1986). Although tentative assumption at that time was that GnRH accomplished the behavioral effect through neuronal activation of the ventral tegmental area, results of our recent behavioral (Hasegawa et al., 1991) and electrophysiological (Hasegawa and Sakuma, 1993) studies suggest otherwise. Inhibition of neuronal activity in the ventral tegmental area caused by microiontophoresis of GnRH, that confirms results of the behavioral study (Suga et al., 1997), will be discussed later in this chapter.

GnRH receptors in the pituitary and brain

Species differences and receptor subtypes

GnRH receptors with characteristics similar to those in the anterior pituitary have been identified in the rat brain. However, as much as 9 subtypes of GnRH, which differ in amino-acid contents and hy-

hypophysiotropic potencies are known between different species (King and Millar, 1995). Mammalian and chicken-II GnRH molecules are co-localized in the musk shrew brain (Rissman et al., 1995). In addition to mammalian GnRH, GnRH-II has been detected in the rat midbrain (Parhar, unpublished observation). Thus, co-localization of different GnRH species in the rat brain appears to be a distinct possibility.

It was noted that the behavioral effect does not require the entire amino-acid sequence of the decapeptide, but a fragment Ac-GnRH⁵⁻¹⁰ possesses a comparable potency as GnRH decapeptide to augment lordosis reflex in estrogen-treated ovariectomized female rats (Dudley and Moss, 1988). In addition to GnRH decapeptide and its C-terminal fragments, several GnRH analogs modulate the lordosis reflex. It has been shown that the behavioral effects of analogs do not always correlate straightforwardly with their hypophysiotropic actions in the female (Dudley et al., 1981; Zadina et al., 1981; Sakuma and Pfaff, 1983; Moss and Dudley, 1990) as well as male (Bhasin et al., 1988) rats. In an *in vitro* system, certain analog stimulates inositol phospholipid turnover and increases intracellular Ca²⁺ as does the decapeptide, but does not induce gonadotropin secretion in pituitary gonadotrophs (Levy et al., 1990).

Some behaviorally active analogs, that inhibit pituitary gonadotropin secretion, often produce agonistic responses when applied to neurons by microiontophoresis. Microiontophoresis of Ac-GnRH⁵⁻¹⁰, which lacks hypophysiotropic activity, induces changes similar to those induced by GnRH decapeptide in the medial amygdala (Dudley et al., 1990), midbrain central gray (Chan et al., 1985; Schiess et al., 1987) and hippocampus (Chen et al., 1993). Microiontophoresis of a fragment without behavioral action to neurons in these structures showed no effect at all.

These observations imply multitudes of GnRH molecules and corresponding receptors in the brain that employs different GnRH molecules as their ligands.

GnRH action on neuronal circuitry for female rat sexual behavior

GnRH and its fragments and analogs have been infused into the medial preoptic area, the ventro-

medial hypothalamus (Dudley and Moss, 1988) or the midbrain central gray (Sakuma and Pfaff, 1980; Riskind and Moss, 1983b; Sakuma and Pfaff, 1983; Sirinathsinghji, 1984; Dudley and Moss, 1988) to study their effects on the lordosis reflex. It is of interest that D-Phe², D-Ala⁶-GnRH, an antagonist analog for pituitary gonadotropin secretion (Beattie et al., 1975), was more potent to promote the lordosis reflex than GnRH decapeptide when applied to the medial preoptic area (Suga and Sakuma, unpublished observation) or the midbrain central gray (Sakuma and Pfaff, 1983) (Fig. 1).

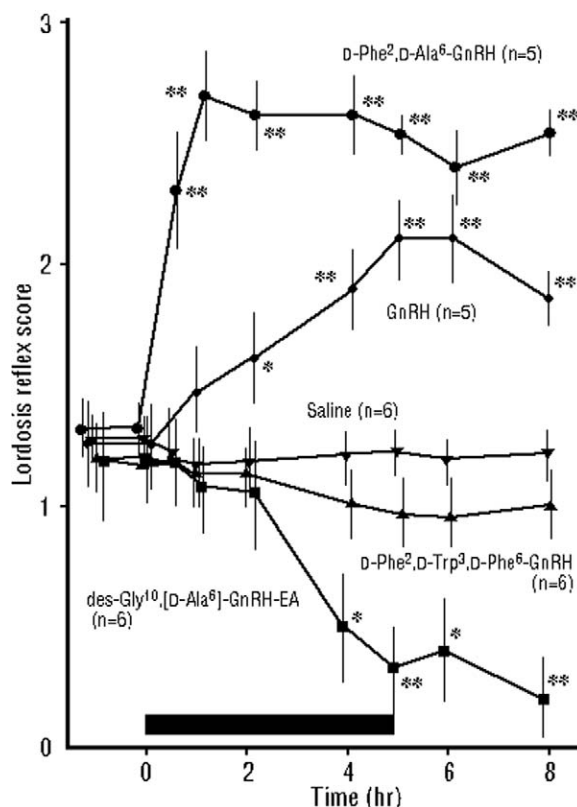


Fig. 1. Time course of changes in the lordosis reflex score induced by GnRH or its analogs in agar-saline gel. Cannulae containing GnRH or analogs were placed in the midbrain central gray for a 5-hour period, as indicated by the solid bar at the bottom. des-Gly¹⁰, [D-Ala⁶]-GnRH-EA is an agonist, and D-Phe², D-Ala⁶-GnRH and D-Phe², D-Trp³, D-Phe⁶-GnRH are antagonists in terms of their effects on pituitary LH release. Vertical bars denote SEM; *n* = number of rats. **p* < 0.05; ***p* < 0.01 when compared to blank (agar-saline) cannula at each time point (two-way analysis of variance and Student-Newman-Keuls test). Figure reproduced, with permission, from Suga et al., 1997).

The restoration of reproductive capability in the hypogonadal mice by implants of preoptic GnRH cells has been attributed to the innervation of the median eminence by axons of the implanted GnRH cells (Gibson et al., 1987; Saitoh et al., 1992). Neural basis for the emergence of the lordosis reflex in these animals remains unknown.

A lesion study implicates the dorsal hippocampus in the facilitation of the lordosis reflex (Cameron et al., 1979). The effect has been attributed to interactions with progesterone-sensitive or serotonergic neural system (Franck and Ward, 1981). However, further scrutiny is needed to establish causal relationship between the GnRH-induced changes in the neuronal activity in the hippocampus (Chen et al., 1993), that presumably depend on GnRH receptors in this structure (Jennes et al., 1988), and the regulation of female rat sexual behavior.

The ventral tegmental area as a relay for the GnRH effect

Electrical stimulation of the midbrain ventral tegmental area disrupted the lordosis reflex without afflicting proceptive components of female rat sexual behavior (Hasegawa et al., 1991) as did the medial preoptic stimulation (Takeo et al., 1993). Although the ascending, primarily dopaminergic projection toward the forebrain is widely acknowledged to be the major route for the ventral tegmental area to accomplish its behavioral and emotional regulation (Kato and Sakuma, 2000), results from both behavioral (Hasegawa et al., 1991) and electrophysiological (Sakamoto et al., 1993) experiments implicate non-dopaminergic (Fallon et al., 1984; Kalen et al., 1988), descending (Beckstead et al., 1979; Swanson, 1982) efferents in the disruption of the lordosis reflex.

We have shown that estrogen decreases the excitability of efferents of the ventral tegmental area that descend in the ventral central gray toward the lower brain stem (Hasegawa and Sakuma, 1993). Furthermore, these ventral tegmental neurons are innervated by preoptic neurons that are similarly inhibited by estrogen (Sakamoto et al., 1993). Taken together, these observations lead to a conclusion that estrogen promotes lordosis reflex by disinhibition of the preoptic and ventral tegmental neuronal links.

The midbrain ventral tegmental area has been

positively associated with GnRH-dependent regulation of the female rat sexual behavior. GnRH infusion into the ventral tegmental area potentiates the lordosis reflex. The effect persists in animals with neurotoxic deletions of dopaminergic neurons (Sirinathsinghji et al., 1986). The GnRH-induced facilitation of the lordosis reflex, therefore, does not depend on dopaminergic neurons as in the stimulus-bound inhibition of the reflex in the pimozide-treated animals (Hasegawa et al., 1991). The opposite effects of GnRH infusion and local electrical stimulation suggest that GnRH suppresses the activity of the descending, estrogen-sensitive neurons in the ventral tegmental area.

Microiontophoresis of GnRH and its behaviorally active analog *D*-Phe², *D*-Ala⁶-GnRH in the ventral tegmental area support this theory (Fig. 2). These substances predominantly suppress the activity of neurons that are antidromically activated from the central gray, in which axons of non-dopaminergic neurons in the ventral tegmental area have been shown to descend toward the lower brain stem (Beckstead et al., 1979).

Neuronal sensitivity to GnRH depends critically on estrogen. In the estrogen-primed ovariectomized rats, 8 identified neurons were inhibited by GnRH without exception, whereas GnRH induced no inhibition and excited 3 among 10 identified neurons in the ovariectomized rats without estrogen treatment. The behaviorally active analog *D*-Phe², *D*-Ala⁶-GnRH, that enhances lordosis reflex, inhibited 3 and excited other 3 neurons in the estrogen-treated ovariectomized rats, but lacked both behavioral and neuronal effects in the absence of estrogen. Other neural effects of estrogen in the ventral tegmental area include a decreased spontaneous discharge rate and an increase in the antidromic activation threshold, both of which bolster the theory that inhibition of neuronal transmission via the ventral tegmental area may be causally related to the enhancement of the lordosis reflex; GnRH and estrogen-regulated expression of GnRH receptors may mediate the inhibitory effects of estrogen.

Conclusion

Since GnRH was first described in 1970s as a neuronal modulator in the regulation of the lordosis

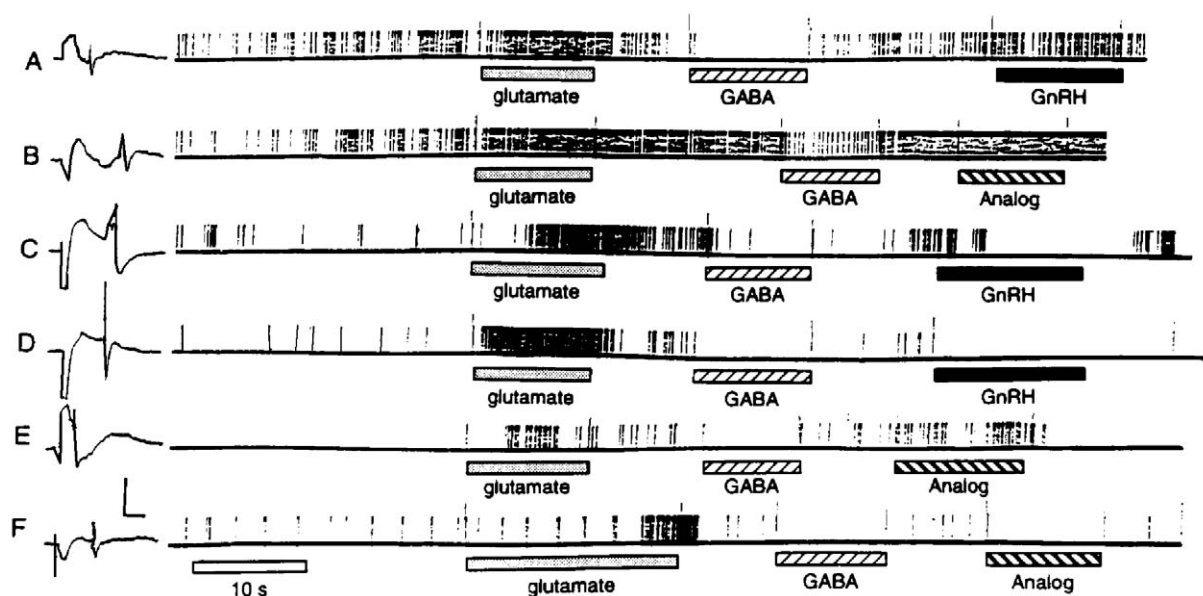


Fig. 2. Antidromic action potentials and response profiles of microiontophoresis of 6 representative neurons. Neurons in ovariectomized non-treated rats had higher firing rates and showed no response to GnRH (A) and to D-Phe²,D-Ala⁶-GnRH (Analog) (B). (C–F), recordings from ovariectomized, estrogen-primed rats. Typical GnRH-induced inhibition of the spontaneous firing in two identified neurons (C, D), with different time courses. (E), a 'silent' neuron which was activated by the Analog; (F), an excitation caused by the analog in a different neuron. Calibrations for the antidromic potentials, 5 ms and 5 mV; scale for the chart recordings, 10 s. Figure reproduced, with permission, from Sakuma and Pfaff (1983).

reflex in the female rat in estrus, many other peptides, amines and other molecules have been added to the list of substances that affects this behavior. However, in our understanding of the control of the lordosis reflex, GnRH ranks high among these substances. This applies to our knowledge on both site and molecular form of the neurotropic action. However, some question persists as more answers are provided by behavioral and neurophysiological studies on the roles of GnRH in the regulation of female rat sexual behavior.

Among the issues discussed in this review, it may well be questioned whether the estrogen-sensitive preoptic projections to the ventral tegmental area employ GnRH as neurotransmitter. Although results from behavioral and neurophysiological experiments favor this possibility, it contradicts to an accepted wisdom that GnRH neurons do not express ER α . GnRH neurons with midbrain projections may make an exception for this consensus, or GnRHergic interneurons in the midbrain, that evade neurophysiological analysis, may intervene. Another possibility is that the estrogen effects may be accomplished via

ER β or through non-genomic routes. Co-localization of ER β and GnRH has been shown in certain preoptic neurons (Skynner et al., 1999a; Kallo et al., 2001). An emerging hypothesis implicates the binding of estrogen to presumed membrane receptors, or interactions with the binding of ligands to their receptors (Raab et al., 1999; Clarke et al., 2000). Experiments are being undertaken to determine possible interactions between GnRH and estrogen via these routes.

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CHAPTER 22

Nutrition, reproduction, and behavior

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Introduction

The relationship between nutrition and reproduction has been demonstrated in species from every mammalian order, including primates (Wade et al., 1996). Successful pregnancy and lactation require tremendous amounts of energy. Ovulatory cycles, mating behavior, steroidogenesis, and lactation are inhibited by caloric restriction, especially in small mammals (reviewed in Schneider and Wade, 2000). This reproductive inhibition is caused by negative energy balance. For example, females who exercise excessively without a compensatory increase in food intake often become amenorrheic (Sanborn et al., 1982; Williams et al., 2001). Negative energy balance inhibits reproduction and available energy is allocated to immediate survival needs, such as maintaining body temperature, metabolism, and foraging (Bronson, 2000; Fig. 1). This insures that reproduction is timed to coincide with resource abundance, which is favorable to survival of offspring.

Interactions between nutrition and reproduction

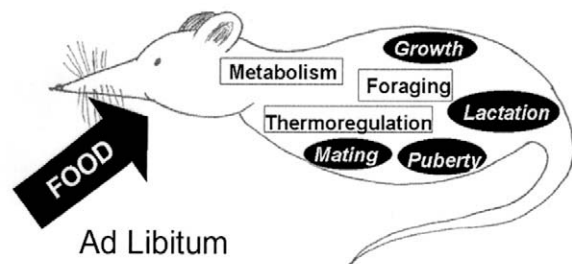
Gonadotropin-releasing hormone

Reproductive inhibition in response to undernutrition correlates with alterations in GnRH pulsatility

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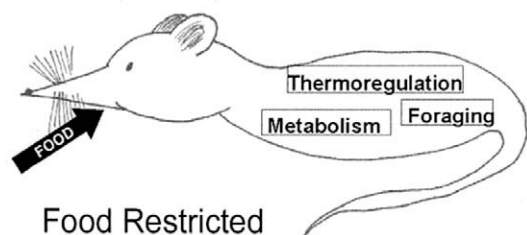


Fig. 1. A schematic drawing of a well-fed (A) and a food restricted (B) musk shrew. Words inside the squares are survival needs and italicized words inside the black ovals are reproductive activities. Notice that when food intake is limited, reproductive activities are suppressed so that available energy can be shifted to meet survival needs.

(reviewed in Schneider and Wade, 2000). Food restriction inhibits the frequency of pulsatile release of GnRH (I'Anson et al., 2000). This decrease in GnRH pulse frequency is correlated with an overall decrease in luteinizing hormone (LH) pulse frequency and a decrease in plasma LH levels (Bronson, 1988;

McGuire et al., 1996; Adam et al., 1997). When glycolysis is blocked, in place of food restriction, LH pulse frequency is also inhibited in sheep (Bucholtz et al., 1996). Conversely, the suppression of gonadotropin release and ovulatory cycles caused by food restriction can be reversed by pulsatile delivery of GnRH (Foster and Olster, 1985; Bronson, 1986; Armstrong and Britt, 1987; Cameron and Nosbisch, 1991; Cameron, 1996). While GnRH pulsatility appears to be one level of regulation, it is probably not the only aspect of GnRH function on which food restriction acts. For example, caloric restriction may affect GnRH production, pituitary sensitivity to GnRH, and/or steroid negative-feedback on GnRH.

In food restricted ewes maintained at a prepubertal weight (growth-restricted), a higher percentage of GnRH-immunoreactive (GnRH-ir) neurons are present in the medial basal hypothalamus (MBH) as compared to *ad libitum* fed lambs (I'Anson et al., 1997). This neuronal distribution is more similar to that of the fetal lamb than the adult. Because food restriction was imposed after birth, it is unlikely that it affected migration of GnRH neurons. Instead, it is likely that the MBH population of GnRH neurons is particularly susceptible to increased activation of inhibitory neurotransmitter pathways, and thus GnRH release is inhibited from cells in this region. A decline in GnRH release could have resulted in peptide accumulation in the GnRH cell bodies. Yet, there were no differences in GnRH content in the MBH between restricted and *ad libitum* fed ewes. Higher levels of GnRH might be expected if there was an accumulation of GnRH in MBH cells and terminals (Ebling et al., 1990). However, one problem with peptide content studies is the loss of cellular resolution. Thus, measurements of peptide content in regions containing both cells and fibers may not reveal more subtle changes in GnRH neurons. A recent study in male prairie voles showed that prolonged (1–3 weeks) food restriction increased GnRH-ir cell number in the POA, GnRH-ir soma size, and GnRH fiber density (Kriegsfeld et al., 2001). These data support the idea that GnRH release is inhibited by food restriction.

In addition to alterations in GnRH peptide content, nutritional manipulations may affect transcription of GnRH mRNA. Gruenewald and Matsumoto

(1993) used in situ hybridization to examine levels of preproGnRH mRNA expression in food deprived (60 hours) and *ad libitum* fed male rats. Food deprived rats had significantly fewer cells expressing GnRH mRNA, but the amount of GnRH mRNA per cell did not differ between the groups. While this study suggests that there are alterations in GnRH mRNA after food restriction, other studies have produced conflicting results. Nappi and Rivest (1997) found no differences in the level of GnRH gene expression per cell between fed and fasted cycling female rats. In fasted female rats GnRH mRNA levels correlated with the presence or absence of estrous cycling. Non-cycling females had lower levels of GnRH expression per cell. This result reveals a relationship between GnRH gene expression and estrous cycling that is not necessarily directly related to nutritional status. A review by Polkowska (1996) also suggests that, although food restriction delays puberty in ewes, when the GnRH-ir neurons are examined, their numbers are normal as is their morphology. Although these data are equivocal, the studies highlighted here suggest subtle effects of food restriction on GnRH production. In addition, differences in GnRH production and regulation after food restriction are species and sex-specific.

Luteinizing hormone

Downstream of GnRH, the HPG axis may be susceptible to caloric restriction via altered LH secretion. Release of LH can be influenced by food intake indirectly due to deficient signaling from the brain, or directly, due to effects on pituitary GnRH receptors, LH or FSH production, and/or gonadotropin release. Food restricted female animals tend to have lower plasma levels of LH and FSH than *ad libitum* fed animals (Bergendahl et al., 1989; Foster et al., 1989). In both men and women, GnRH treatment produced elevated levels of plasma LH and FSH in fasted, but not control, individuals (Rojdmark, 1987). Similarly, ovariectomized (OVX), steroid primed food restricted (60% of *ad lib* intake for 9–11 days) rats had higher plasma LH and FSH in response to low-dose administration of GnRH than *ad libitum* fed controls (Lively and Piacsek, 1988). This increase in pituitary sensitivity to GnRH is interesting and further supports the idea that the GnRH neuron pulse

generator is the main level at which nutrition regulates fertility.

Another measure of food restriction effects on the HPG axis is pituitary LH and FSH content. Female rats maintained on a restricted diet (10 grams of food daily) for 60 days had lower pituitary LH and FSH content, lower serum LH, FSH and E_2 levels, and smaller and fewer gonadotropin positive pituitary cells compared to *ad libitum* fed controls (Kotsuji et al., 1992). These effects were reversed by daily administration of GnRH. Because GnRH corrected the effects of food restriction, it is likely that the primary deficit is in endogenous GnRH production and/or release. Food restricted male rats (60% of *ad libitum* for 6 weeks) had lower anterior pituitary content of LH β and FSH β mRNAs than *ad libitum* fed controls (Hans et al., 1998). Shorter duration of underfeeding (four days instead of six weeks) produced a 42% decrease in pituitary content of the gonadotropin alpha-subunit as compared to *ad libitum* fed controls, but no difference in either of the β subunits were noted (Bergendahl et al., 1989). These contradictory findings could be related to differences in the severity and duration of the food restriction. However, despite the discrepancies in the data, both studies showed that food restriction led to a decrease in pituitary gonadotropin mRNAs. In sum, nutritional manipulations affect multiple levels of the HPG axis, from GnRH production and release to gonadotropin subunit expression and production.

Negative feedback

Negative feedback of steroid hormones on the HPG axis is crucial for its proper functioning. Estradiol and testosterone (T) act at both the pituitary and the hypothalamus to decrease LH release, presumably by decreasing GnRH secretion (reviewed in Brown, 1994). Food restriction can increase sensitivity to steroid negative feedback. Food deprivation for 48 hours in cycling rats decreased plasma LH and lowered the frequency and amplitude of the LH pulse (Cagampang et al., 1990). This same manipulation had no effect in ovariectomized rats, even when the food deprivation was extended to 72 hours (Cagampang et al., 1990). A similar effect was reported in gonad-intact male rats, where food restriction to 50% of *ad libitum* for 7 days decreased LH levels com-

pared to *ad libitum* fed controls. The same restriction paradigm had no effect in castrated rats (Dong et al., 1994). These data suggest that food restriction only inhibits the GnRH pulse generator when gonads are intact. It is possible that the initial release from negative feedback following gonadectomy produces such high levels of LH that acute food restriction is not sufficient to produce notable differences. Bergendahl and Hutaneimi (1994) found that a 5-day fast suppressed LH secretion in sham-operated and acutely castrated rats, but castration 2 weeks prior to food deprivation stimulated LH release. Thus, when gonads are intact, the negative effects of food deprivation dominate, but without steroids present, food deprivation may have stimulatory effects.

Another important component of steroid negative feedback is estrogen action via neural estrogen receptors. Li et al. (1994) demonstrated that ovariectomized hamsters fasted for 48 hours had fewer estrogen receptor immunoreactive (ER-ir) cells in the ventromedial nucleus of the hypothalamus (VMN) and more ER-ir cells in the POA relative to *ad libitum* fed controls. In rats the same effect of acute food deprivation was noted in the VMN (Marin-Bivens et al., 2000). This decrease in ER-ir cells in the VMN probably causes the decrease in lordosis duration after food deprivation, which persists even in the presence of exogenous E_2 (Dickerman et al., 1993). In the POA, an increase in ER-ir cell number may, in part, mediate the increased sensitivity of GnRH to steroids, but at this point, this remains speculation (Li et al., 1994).

Mating behavior

Mating behavior may be the most important level of regulation for reproduction. An animal must mate in order to reproduce, thus, inhibiting mating behavior is the ultimate way to suppress reproduction during unfavorable environmental conditions. In species with estrous cycles, behavioral inhibition can be indirect, via decreases in circulating steroid hormone levels. However, the neural mechanisms, such as regulation of steroid receptor containing cells, that control mating behavior can also be influenced by undernutrition. In food deprived female Syrian hamsters, the sexual posture, lordosis, can be elicited by treatment with estradiol and progesterone, however

the duration of lordosis is significantly shortened by food deprivation relative to *ad libitum* fed females (Dickerman et al., 1993). This same behavioral inhibition can be elicited by pharmacological blockade of metabolic pathways in place of food deprivation. In ovariectomized female hamsters treated with estradiol and progesterone, female mating behavior is disrupted when glycolysis and fatty acid oxidation are blocked simultaneously (Dickerman et al., 1993; Jones and Lubbers, 2001). Perhaps exogenous steroid hormone treatment cannot completely reverse the effects of metabolic fuel limitation on mating behavior because of the simultaneous decrease in estrogen receptor containing cells in the VMN. Thus, in addition to affecting various aspects of the HPG axis, undernutrition also inhibits mating behavior.

Gaps in our knowledge

One important gap in the literature reviewed is the identity of the primary signals from food that trigger the cascade of downstream events that eventually culminate in adjustments in reproductive status to match available energy (Schneider and Wade, 2000). There are many putative factors ranging from metabolic fuels and gut peptides to hormones and neurotransmitters. Some appear to be more relevant than others and there is variation among species in their relative importance (reviewed in Kalra and Kalra, 1996). In addition to not knowing what the signal is, there is also some question about the brain area(s) where these peripheral signals act. The hypothalamus is obviously one area that is affected by undernutrition and in addition to regulating sex behavior, this region plays a role in food intake, motor activity, and ingestive behavior (Williams et al., 2000). Perhaps the hypothalamus is directly sensitive to nutritional status, however, there may be other brain regions, such as the hindbrain, that detect signals reflecting metabolic fuel availability first and then relay information to the hypothalamus (Schneider and Zhu, 1994; Schneider et al., 1995; Grill et al., 1998).

Another aspect of nutrition and reproduction that has not been explored is the GnRH pulse generator. Many of the studies mentioned here show that GnRH pulsatility is affected by undernutrition. This could be due to changes in GnRH production, in GnRH re-

lease, and/or GnRH pulse generation. One aspect yet to be explored is the effect that undernutrition may have on electrical activity of GnRH neurons. With the engineering of transgenic mice that have green fluorescent protein-labeled GnRH neurons; visualization and recording from these cells is simplified (Suter et al., 2000).

Many alterations in the GnRH system after nutritional manipulation have been well-documented. To date, however, no one has linked these changes to behavioral deficits. There is a large literature on extrapituitary effects of GnRH. Intracerebroventricular GnRH administration to ovariectomized/hypophysectomized female rats facilitates sexual receptivity (Pfaff, 1973; Moss and Foreman, 1976). In addition, only 6 amino acids from the GnRH decapeptide are necessary for these effects (Dudley and Moss, 1988; Fernandez-Fewell and Meredith, 1995). What remains to be determined is if the deficits in mating behavior are functionally related to changes in GnRH following food restriction.

The musk shrew

Musk shrews display mating-induced ovulation. Our laboratory has been working with this animal model for over 15 years in order to study environmental regulation of reproduction. Two of the primary stimuli that affect reproduction in this species are mating (Rissman, 1992) and nutritional status (Gill and Rissman, 1996; Temple and Rissman, 2000a). Because both puberty and ovulation are induced by mating, we can precisely control these two neuroendocrine events and study how they are influenced by food manipulations.

Musk shrews have several characteristics that allow us to answer questions that would be difficult using a rodent model. First, they are induced ovulators and do not display any hormonal or behavioral estrous cycles. Instead females usually mate and ovulate whenever they come in contact with a male (Dryden, 1969). Thus, we can use gonad-intact females for our studies because ovaries are in a quiescent state until multiple matings occur. Second, mating behavior is not dependent on high circulating levels of steroid hormones in females. Unlike rodents, plasma E₂ is nearly undetectable prior to mating in this species (Fortune et al., 1992). Instead,

testosterone (T) is the primary steroid hormone secreted from musk shrew ovaries and adrenal glands (Rissman and Bronson, 1987). Although ovaries and adrenals must be intact for mating to occur, the levels of T do not change over a cycle as steroid hormones do in other species (Rissman and Bronson, 1987; Fortman et al., 1992). This offers two distinct advantages. First, experimental manipulations can be applied without concern for disruption of cyclic secretion of hormones. This also allows us to look at direct effects of environmental factors on behavior, instead of indirect effects on behavior due to changes in plasma concentrations of steroid hormones. Second, musk shrews are very sensitive to small reductions in food intake and respond rapidly to reinstatement of food after a mild food restriction (Gill and Rissman, 1996; Temple and Rissman, 2000a). This allows us to examine primary metabolic signals that influence mating behavior and HPG axis function.

Behavioral regulation of GnRH

Mating-induced ovulation is a reproductive strategy that is used by females of many species. In most well-studied species, the female comes into estrus, which is characterized by high plasma levels of estradiol. Estrus is stimulated by environmental cues, such as photoperiod in ferrets and rabbits, or

contact with male conspecifics in voles (reviewed in Spies et al., 1997). Once plasma estradiol levels are high, females will mate and mating induces ovulation. Mating induced ovulation in the musk shrew has several features that differ from the 'classic' case described above. First, mating is not dependent upon high levels of estradiol, thus female shrews do not come into estrus, per se (Rissman et al., 1987; Rissman and Crews, 1988). Instead, female musk shrews mate whenever they come in contact with a male, despite having undetectable levels of plasma estradiol (Rissman et al., 1987; Fortune et al., 1992). Second, musk shrews rarely become pregnant after their first mating bout (Clendenon and Rissman, 1990; Rissman, 1992). Instead, two or three matings, separated by at least 24 hours, are required to induce ovulation (Rissman, 1992).

Mating has a significant effect on GnRH production in the female musk shrew. GnRH content increases in forebrain nuclei 24–48 hours after the initial mating bout (Dellovade et al., 1995b; Fig. 2). In addition, if this mating bout does not result in ovulation, GnRH content remains high until the female copulates again. Once the female ovulates, GnRH content drops significantly, suggesting that GnRH has been released. In addition, the number of forebrain GnRH immunoreactive cells is increased after mating as well as after brief interactions with males that do not include mating (Dellovade and Rissman,

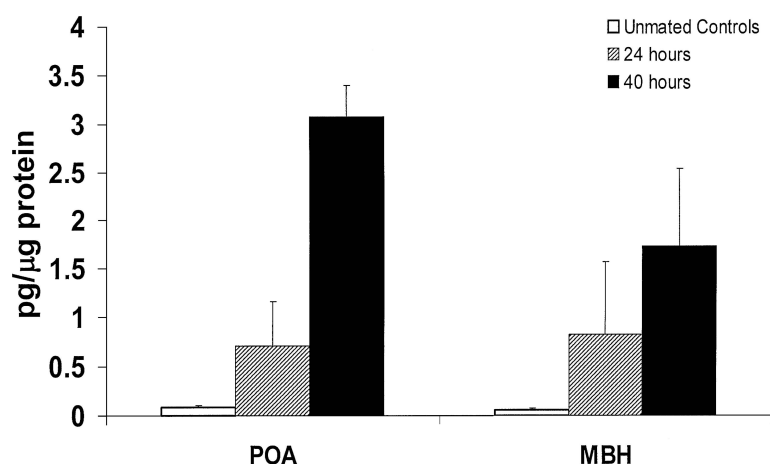


Fig. 2. Mean \pm SEM amount of GnRH (pg/ μ g protein) in neural tissue taken from the preoptic area (POA) and the medial basal hypothalamus (MBH) from females who either received no mating or were sacrificed 24 or 40 hours after a single ejaculation was received. Redrawn from Dellovade et al., 1995b.

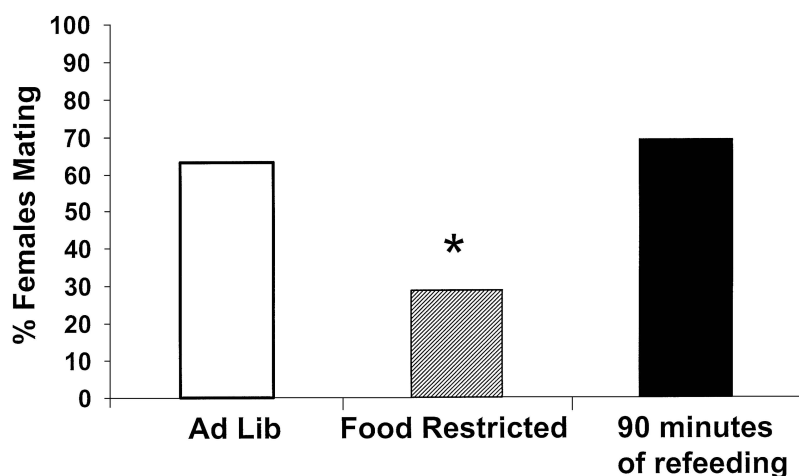


Fig. 3. Percentage of females receiving 5 placed intromissions during a mating test. Subjects were either fed *ad libitum* ($n = 16$), food restricted to 60% of *ad libitum* ($n = 15$), or food restricted to 60% of *ad libitum* and re-fed 90 minutes prior to the mating bout ($n = 15$). * = significantly different from other groups ($p < 0.05$). Redrawn from Temple and Rissman, 2000a.

1994; Dellovade et al., 1995a,b). These data suggest that mating directly regulates several aspects of GnRH system functioning, including GnRH production and/or release.

Nutritional regulation of mating behavior

Because mating behavior is integral for regulation of ovulation and GnRH in this species, and because it has been well-established in other species that successful reproduction depends on sufficient food availability, we investigated the consequences of nutritional manipulation on mating behavior in female musk shrews. In one study 48 hours of food-restriction significantly decreased the percentage of females displaying mating behavior (Gill and Rissman, 1996). Next, we focused on recovery of function after food restriction. Because female musk shrews do not rely on peak steroid hormone levels for mating, we hypothesized that restoration of mating behavior after food restriction would occur rapidly. We examined mating behavior in female shrews that were either fed *ad libitum*, food restricted to 60% of their average *ad libitum* food intake for 48 hours, or food restricted as just described, but given *ad libitum* access to food 90 minutes prior to mating. We found that food restriction significantly reduced the percentage of females that displayed mating behavior (Temple and Rissman, 2000a). More importantly,

this behavioral deficit was completely reversed by only 90 minutes of re-feeding (Fig. 3). These effects of nutritional manipulation on mating could have been due to either changes in circulating steroid hormone levels and/or changes in androgen receptor or estrogen receptor immunoreactive cell numbers. We have shown that neither of these is the case. Food restriction and re-feeding had no effect on plasma testosterone concentrations. Moreover, testosterone administration was ineffective in reversing the behavioral effects of food restriction (Temple and Rissman, 2000a).

Although plasma steroid hormone concentrations were not affected by nutritional manipulation, it is possible that neural steroid hormone receptors could have been. Food deprivation decreases the number of ER-ir cell in the VMN of female hamsters (Li et al., 1994). In order to explore this hypothesis, we counted the number of ER-ir and androgen receptor immunoreactive (AR-ir) cells in the POA and VMN of females that were either fed *ad libitum*, food restricted for 48 hours, or food restricted and re-fed for 90 minutes prior to sacrifice. There were no differences in any of these conditions in numbers of ER-ir or AR-ir cells in either the POA or VMN (Temple and Rissman, 1999; Figs. 4 and 5). These findings lead us to conclude that there is an alternative, non-steroidal mechanism by which nutritional manipulations affect mating behavior.

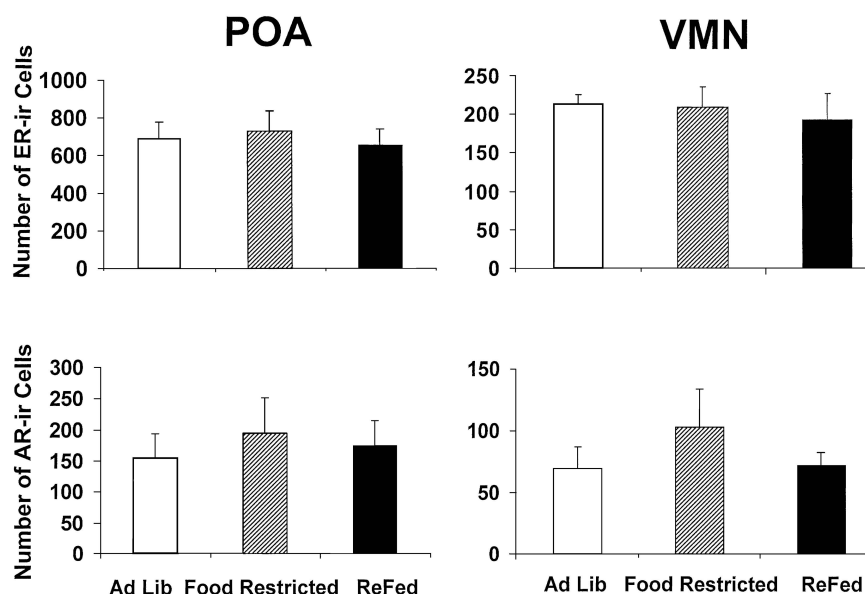


Fig. 4. Mean \pm SEM number of estrogen receptor immunoreactive (ER-ir; top panel) and androgen receptor immunoreactive (AR-ir; bottom panel) cells in the preoptic area (POA; left panel) and the ventromedial nucleus of the hypothalamus (VMN; right panel). Females were either fed *ad libitum*, food restricted to 60% of *ad libitum*, or food restricted to 60% of *ad libitum* and re-fed 90 minutes prior to the mating bout ($n = 8$ per feeding condition). There were no significant differences among any of the feeding conditions in either area of the brain.

These data demonstrate that mating behavior can be influenced by acute metabolic fuel availability. The rapidity of behavioral recovery in this species is faster than anything reported in the literature. In addition, because these effects are occurring in the absence of changes in steroid hormone concentrations or neural steroid hormone receptors, the musk shrew is a good model species for the study of non-steroidal mediators of nutritional infertility. Because mating behavior is a salient, non-invasive measure of reproduction, this model is also excellent to assess primary signals from food that affect reproduction.

Metabolic cues and mating behavior

One unanswered question in this field is which primary signals, associated with food, initiate and support reproductive processes? We hypothesized that signals generated by the oxidation of metabolic fuels were being utilized to reinstate mating behavior in musk shrews. To test this hypothesis, we blocked individual metabolic fuel pathways during the 90 minute refeeding period and examined the effects on behavioral recovery of mating behavior. When

either glycolysis or fatty acid oxidation were inhibited individually, the refeeding-induced restoration of mating behavior was suppressed (Temple et al., 2002). Therefore both glycolysis and fatty acid oxidation are necessary for behavioral recovery. Next, either glucose or fat were given to food restricted females to determine whether either metabolic fuel substrate could support mating after food restriction. We found that neither fuel alone was sufficient (Temple et al., 2002). These data suggest that glucose and fatty acids are likely primary signals for mating behavior. In addition, because both fuels appear to be necessary simultaneously, it is likely that they are both acting to increase the levels of a common signal (such as ATP) that lies downstream of metabolic fuel oxidation.

Nutritional regulation of GnRH

To determine if nutritional status has direct effects on GnRH, we examined three aspects of GnRH system function in females from each of our feeding conditions (*ad libitum* fed, food restricted, and food restricted and re-fed for 90 minutes). First, we quan-

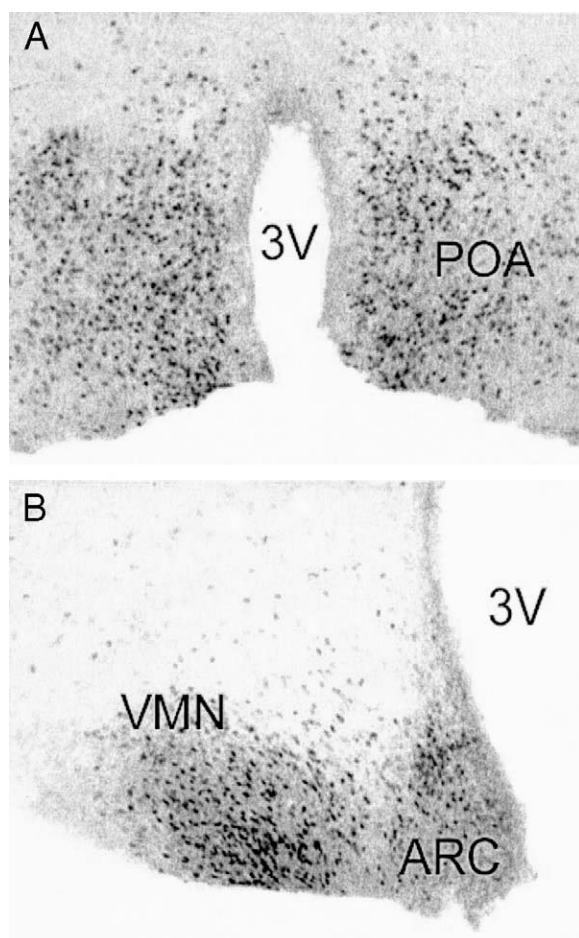


Fig. 5. Photomicrographs of estrogen receptor immunoreactive staining in the preoptic area (A) and the ventromedial nucleus of the hypothalamus (B). Photographs were taken at 20 \times magnification. 3V = third ventricle, POA = preoptic area, ARC = arcuate nucleus, VMN = ventromedial nucleus of the hypothalamus.

tified proGnRH immunoreactive neurons in several areas of the forebrain. Food restricted females had significantly more proGnRH-ir cells in the POA than either the *ad libitum* fed or re-fed females (Temple and Rissman, 2000b; Fig. 6). One interpretation of these data is that food restriction decreases the rate at which proGnRH is processed to the GnRH decapeptide, thus increasing the amount of peptide available for detection by immunocytochemistry. In addition, because the re-fed group of females had numbers of immunoreactive cells that were comparable to *ad libitum* fed females, we hypothesized that re-feeding

increases the rate of GnRH processing. There are at least four enzymatic processing steps involved in the conversion of proGnRH to the mature and functional decapeptide form (Wetsel et al., 1995). Each of these enzymes has the potential to modify GnRH abundance in response to nutritional signals. We have also examined the effects of nutritional status on GnRH content in the POA and in the medial basal hypothalamus. Levels of GnRH peptide are uniform in the POA regardless of feeding condition. Yet, food restricted females had a 30-fold increase in the amount of GnRH present in the MBH as compared to *ad lib* and re-fed females (Temple and Rissman, 2000b; Fig. 7). This suggests that food restriction inhibits GnRH release from its terminals, and that re-feeding rapidly reverses this effect. We also examined the effects of nutritional status on pituitary responsiveness to exogenous GnRH. Nearly 100% of the females in all feeding conditions ovulated 20–24 hours after GnRH treatment. However, food restricted females had significantly fewer corpora lutea, suggesting significantly fewer ova ovulated, as compared to *ad libitum* and re-fed females (Temple and Rissman, 2000b). In the musk shrew food restriction affects every level of the HPG axis that we examined. This is not surprising and, in fact, this corresponds very well with what we know from other species. What was surprising is that in this species, the HPG axis deficits brought on by food restriction were all reversed after 90 minutes of re-feeding.

Future directions

Currently, we are examining the link between the undernutrition-induced behavioral deficits and the GnRH system. We have shown that undernutrition decreases the availability and/or release of GnRH, and also inhibits mating behavior. Because GnRH has effects on mating behavior in both male and female mammals (Zadina et al., 1981; Fernandez-Fewell and Meredith, 1995), we have asked if these two consequences of food limitation are related. In the musk shrew, intracerebroventricular (i.c.v.) administration of mammalian GnRH facilitates sexual receptivity in females. Administration of a GnRH antagonist into the third ventricle of ovariectomized, steroid-primed rats inhibits lordosis (Dudley et al., 1981; Moss and Dudley, 1990). In addition, GnRH

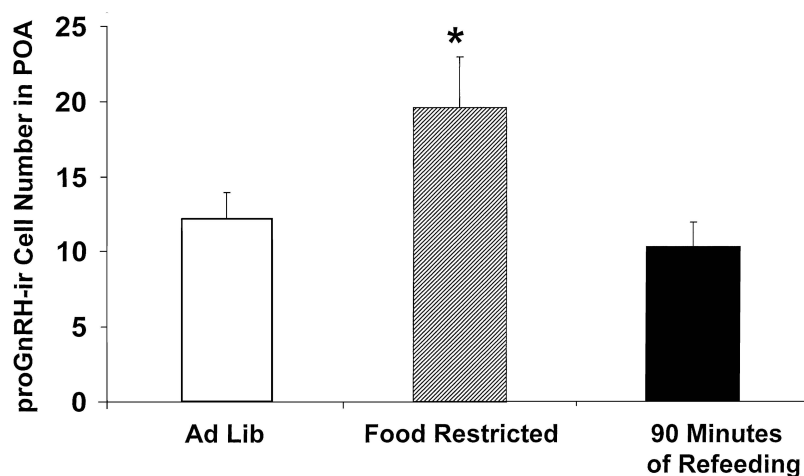


Fig. 6. The mean \pm SEM number of proGnRH immunoreactive (proGnRH-ir) cells in the preoptic area (POA) of females who were either fed *ad libitum* ($n = 6$), food restricted to 60% of *ad libitum* ($n = 7$), or food restricted to 60% of *ad libitum* and re-fed 90 minutes prior to the mating bout ($n = 7$). * = significantly different from other groups ($p < 0.05$). Redrawn from Temple and Rissman, 2000b.

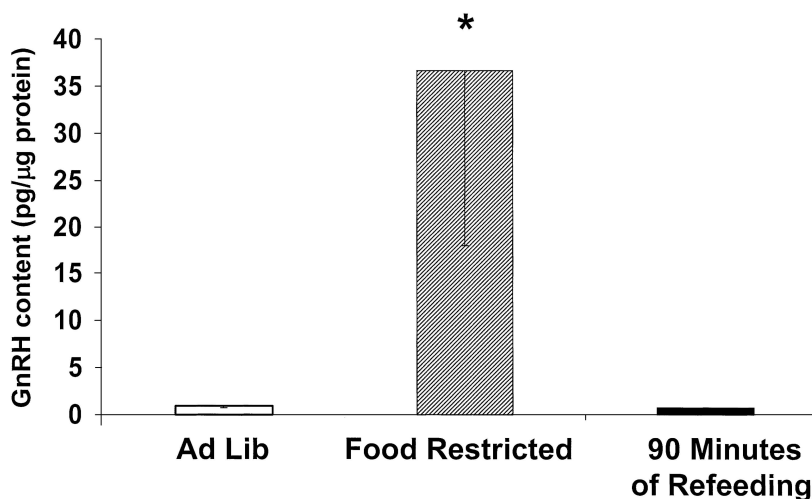


Fig. 7. Mean \pm SEM amount of GnRH (pg/ μ g protein) in neural tissue taken from the medial basal hypothalamus (MBH) from females who were either fed *ad libitum* ($n = 11$), food restricted to 60% of *ad libitum* ($n = 11$), or food restricted to 60% of *ad libitum* and re-fed 90 minutes prior to the mating bout ($n = 13$). * = significantly different from other groups ($p < 0.05$). Redrawn from Temple and Rissman, 2000b.

administration to male hamsters can elicit mating behavior after the removal of the vomeronasal organ (Fernandez-Fewell and Meredith, 1995). We hypothesize that i.c.v. administration of GnRH to food restricted musk shrews will restore mating behavior. While several studies have shown that GnRH release and mating behavior are both affected by nutritional manipulation, ours would be the first to show a functional relationship between these two deficits.

Lessons to be learned from the musk shrew

The strength of the musk shrew model lies in our ability to precisely control the onset of puberty. Because of this, we can examine the effects of environmental stimuli on pubertal maturation with a high degree of precision. In terms of nutritional infertility, the advantages are two-fold. First, we can examine steroid-independent effects of nutrition on

both mating behavior and on properties of GnRH neurons in gonad-intact females. In other species, food restriction decreases steroidogenesis, thus the kinds of studies we do would have to be done in ovariectomized, steroid-treated females. This type of manipulation is useful, but there are limitations on how results are interpreted and their ecological relevance. Second, because restoration of reproductive function occurs rapidly in musk shrews, we can examine the primary signals from food intake that affect mating behavior and the HPG axis during a finite time period. This is a problem that is unsolved in part because behavioral restoration after food restriction takes a minimum of 6 hours after re-feeding, even in steroid treated females (Jones and Lubbers, 2001). After this much time has passed, primary signals have already acted on multiple downstream targets.

Our data show that the musk shrew is a good model for examining how nutrition affects reproduction. Musk shrews are extremely sensitive to small and rapid fluctuations in food intake. In addition, because we can work with gonadally intact animals, we can examine the consequences of nutritional manipulation in a physiologically relevant manner. The studies highlighted here provide a comparative perspective on mechanisms underlying nutrition and reproduction interactions in mammals.

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GnRH, brain mast cells and behavior

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Introduction

Mast cells, potent unicellular migratory glands, are found in all tissues and organs of the body including the brain and reproductive tract. In this chapter we will show that GnRH is found in mast cell granules. We will also present evidence that the brain mast cell population increases under specific behavioral and endocrine states, including those associated with reproduction. Where pertinent we will also discuss other mast cell–neuroendocrine interactions.

Mast cells are derived from the haematopoietic stem cell of the bone marrow. They circulate as committed precursors and undergo their final phenotypic differentiation in a tissue specific manner. Progenitors of mast cells contain cytoplasmic granules, and express RNAs encoding mast cell-associated proteases, but lack expression of the high-affinity immunoglobulin E receptor (Rodewald et al., 1996). In the ring dove, immature mast cells were identified at the ultrastructural level as early as embryonic day 15. At this age, the cells are present in the pia adjacent to the medial habenula. They have fewer granules which are more electron lucent compared to granules in mature cells (Fig. 1A and B). The staining properties of mast cells change with maturation, reflecting changes in the content of the granules (Zhuang et al.,

1999). Mast cells are highly plastic in that they can change phenotype when transplanted from one environment to another (e.g., from gut mucosa to connective tissue; Kitamura et al., 1987a,b). Depending on their place of residence, alterations in environmental signals and activational state, mast cells store or produce upon activation, a wide range of mediators. Stored classes of mediators include: biogenic amines, bioactive peptides (including gonadotropin releasing hormone (GnRH)), mast cell specific serine proteases, and heparin sulfated proteoglycans. Interestingly, mast cells are the only source of heparin in the body (Humphries et al., 1999). Upon stimulation by immune or non-immune signals they can make and release prostaglandins, leukotrienes, cytokines and growth factors to list but a few of the potential categories (reviewed in Wedemeyer et al., 2000).

Mast cells are long-lived cells and can undergo many rounds of activation, involving degranulation (Fig. 2, see page 319) and replenishment (Dvorak, 1989; Wilhelm et al., 2000; Xiang et al., 2001). Mast cells can release mediators by piecemeal degranulation (Kops et al., 1990; Dvorak, 1992; Wilhelm et al., 2000) resulting in differential release of granular contents. Mast cells also degranulate by compound exocytosis. This involves the fusion of the secretory granule with the plasma membrane, followed by the subsequent fusion of other granules to the first, resulting in the rapid release of soluble granular contents. When this occurs the soluble mediators (e.g., 5-HT) and about 25% of the sulfated proteoglycans diffuse out into the extracellular space. The remain-

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ing material is an insoluble core of heparin sulfated proteoglycans to which the mast cell specific neutral proteases such as chymase (a chymotryptic endopep-

tidase) and tryptase (a trypsin-like exopeptidase) are attached (Kaartinen et al., 1995). Molecules in the mast cell's environment that can bind to heparin and/or to the proteoglycan can be captured by the mast cell, as is the case for such diverse substances as low density lipoprotein (LDL) (Kovanen, 1991) and the chemotherapeutic drug, adriamycin (Crivellato et al., 1997).

Historically there was considerable controversy over the existence of brain mast cells, particularly in the normal human brain (Olsson, 1968). However, mast cells are morphologically and histochemically distinct from other cell types. The occurrence of mast cells in the brain is now indisputable (Dropp, 1976; Ibrahim et al., 1979; Theoharides, 1990; Silver et al., 1996), and a detailed analysis of optimal conditions for visualization of mast cells is available (Florenzano and Bentivoglio, 2000). We have examined the staining properties of brain mast cells in rats, and found that mast cells can be identified with the following 3 markers we have used: serotonin, heparin, and toluidine blue (Fig. 3, see page 319).

GnRH and brain mast cells

Multiple forms of the GnRH decapeptide have been found in the brains of vertebrates. These include the mammalian form, mGnRH (Schally et al., 1971) produced in neurons originating from the mammalian olfactory placode (Schwanzel-Fukuda and Pfaff, 1989; Wray et al., 1989), chicken GnRH-I (cGnRH-I) which differs at position 8 in which glutamine is substituted for an arginine (Gln⁸) (avian placodal form), and chicken GnRH-II (cGnRH-II)

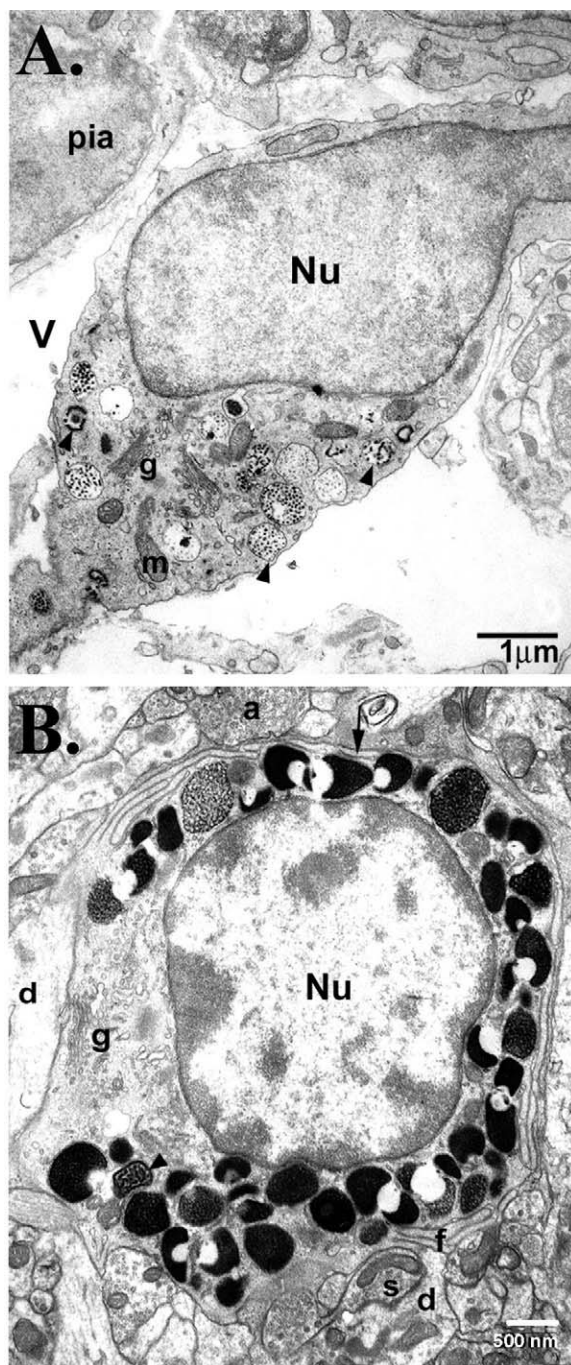


Fig. 1. Electron micrographs of dove brain mast cells. (A) Mast cell in the pia near the choroid plexus of the third ventricle (V) from an E15 dove embryo. Note the elongate shape indicative of cellular migration. Immature granules, with particulate interiors of varying architecture, are indicated with arrowheads. These granules do not contain GnRH nor do they stain with acidic toluidine blue. Nu = nucleus; g = Golgi apparatus. Scale bar = 1 μ m. (B) By one month of age, the mast cell granules (arrow) are more mature. They are mostly electron dense with an electron lucent cap typical of avian mast cells. Most cells however do not make GnRH at this time. Note that the mast cell is fully integrated into the brain's Nu = nucleus; g = Golgi apparatus. Scale bar = 500 nm.

which contains amino acids His⁵-Trp⁷-Tyr⁸. Antibody absorption studies in dove brain mast cells indicated that mast cell GnRH-like immunoreactivity was abolished when an antibody for mammalian GnRH (LR-1) is preabsorbed with either cGnRH-I or cGnRH-II, while GnRH neurons staining is abolished by cGnRH-I but not cGnRH-II. This suggested that in the dove, the GnRH in mast cells might be a different peptide than that found in GnRH producing neurons.

To test if mast cells could accumulate GnRH via endocytosis, *in vivo* radiolabeled GnRH was infused into the CSF of the dove (Silverman et al., 1994). Mast cells in the pia mater contacting the CSF as well as those in the medial habenula which borders the CSF were not labeled. The negative data are not likely due to a deficiency in endocytosis as mast cells are capable of endocytosis of many substances including synthetic beads, bacteria, and LDL both *in vivo* and *in vitro* (Bacteria — Shin et al., 2000; LDL — Wang et al., 1995). In fact, GnRH immunoreactivity in dove brain mast cells is localized to the Golgi apparatus as well as the secretory granules suggesting *de novo* synthesis (Fig. 4). As noted below, rat mast cells contain GnRH (Fig. 5, see page 319) and preliminary studies in the laboratory suggest that they express GnRH mRNA. We are exploring whether the gene transcript produced is the same as the one found in the hypothalamus.

The presence of GnRH in mast cells is of interest in that the decapeptide has multiple functions besides regulating the pituitary-gonadal axis. It is known to potentiate sexual behavior (Moss and McCann, 1973; Pfaff, 1973) and may function as a neurotransmitter within the brain (Chen et al., 1993). Depending on the species GnRH axons can be found in many brain regions (e.g., amygdala) outside of the septo-hypothalamic pathway to the median eminence (Silverman, 1988). GnRH can also act as a paracrine/autocrine factor and as an immunomodulator (reviewed in Marchetti et al., 1996). Given that mast cells degranulate in the normal brain it is possible that GnRH from this cellular source is active in the CNS.

Other immune cells also synthesize bioactive GnRH peptide and mRNA (Emanuele et al., 1990; Azad et al., 1991; Maier et al., 1992) and GnRH-receptor mRNA (Weesner et al., 1997; Chen et al.,

1999). GnRH has a stimulatory effect on lymphocyte proliferation (Marchetti et al., 1989; Morale et al., 1991) and GnRH signaling at the lymphocyte stimulates expression of the interleukin-2 receptor (Batticane et al., 1991; Chen et al., 1999). In addition, GnRH antagonist administration to mice prone to lupus erythematosus decreases antibody production and hematuria, and increases the animal's life span, suggesting that this autoimmune disease is, in part, attributable to excessive expression of immune cell GnRH (Jacobson et al., 1994). Interestingly GnRH and GnRH agonists and antagonists can induce histamine secretion from mast cells, leading to cutaneous anaphylaxis (Rivier et al., 1986; Phillips et al., 1988; Sundaram et al., 1988). This was a clinical problem in the treatment of precocious puberty, which was solved by the redesign of GnRH analogues (Xiang et al., 2001).

Brain mast cells change with the animal's state

The central nervous system with its formidable blood-tissue barrier is an immunologically privileged site. However, this does not mean that the adult brain escapes immune surveillance. For example, activated T cells enter the normal CNS without precipitating an inflammatory response (Hickey, 2001). Similarly, mast cells enter the normal CNS during development (Lambracht-Hall et al., 1990; Zhuang et al., 1999) and new recruitment can be elicited under normal physiological conditions (Zhuang et al., 1993; Yang et al., 1999). Unlike T cells that apparently enter at random sites in the CNS (Hickey, 2001), mast cells are located in specific brain regions in a species specific manner and, as described below, alterations in their number also occur in specific brain regions and nuclei within those regions.

Brain mast cells and reproduction

A correlation between brain mast cells and reproduction was noted by Flood and Kruger (1970) when they found an increase in the number of mast cells (identified by their metachromatic properties to acidic aniline dyes such as toluidine blue) in the brains of hedgehogs during hibernation, a time of gonadal regression, compared to the awake state. Observations from our laboratory made the first di-

rect connection between mast cell number, their activation state, and the animal's reproductive status. In the ring dove, mast cells are found in several brain regions but are concentrated in the epithalamus (medial habenula) (Zhuang et al., 1999). These were first identified by the presence of GnRH-like immunoreactivity (Silver et al., 1992); mast cell identity was later confirmed by the presence of the biogenic amine, histamine, and by ultrastructural characteristics, especially granular morphology and nuclear substructure (Silverman et al., 1994).

In subsequent experiments in the ring dove we concentrated our observations on the medial habenula as mast cell number and activation are reproducibly associated with sex behavior or sex steroids. Courtship in the ring dove activates ovarian follicular development and estrogen–progesterone secretion in females and stimulates androgen production in males (Feder et al., 1977; Silver et al., 1980). Within two hours of pairing with an adult female, GnRH positive mast cells increase dramatically in number in the medial habenula from ~300 to about ~1200 (Zhuang et al., 1993). Similar observations were made in females though the number of brain mast cells was more variable among females than among males (unpublished data from the laboratory). In males the lowest number of mast cells in the habenula was found in castrated birds (Zhuang et al., 1993). Treatment of birds with silastic capsules containing gonadal steroids (testosterone or dihydrotestosterone

in males, estradiol 17- β in females) also resulted in an elevation in mast cell numbers in the medial habenula (Wilhelm et al., 2000). These findings indicate that the mast cell population in the habenula is elevated by gonadal hormones even in the absence of sexual behavior. There was no sexually dimorphism in the response. There was no difference between testosterone and dihydrotestosterone-treated males indicating that the elevation of mast cell numbers did not require aromatization of androgen in the male. Although the medial habenula has been associated with arousal, its role in reproduction per se is unknown. Nonetheless, specific behaviors and hormonal conditions create states favorable for 'entry' of mast cells into this nucleus (see below).

An increase in the adult brain mast cell number is not confined to avian species. Yang et al. (1999) found that mating followed by cohabitation with females resulted in an increase in mast cells positive for GnRH in the brain of the male mice, with the largest increase observed when males were housed with females for 15 to 19 days post-coitum. In the mouse, mast cells are primarily localized at perivascular sites within selected thalamic nuclei including the ventral posterior, lateral dorsal, central medial and lateral and medial geniculate.

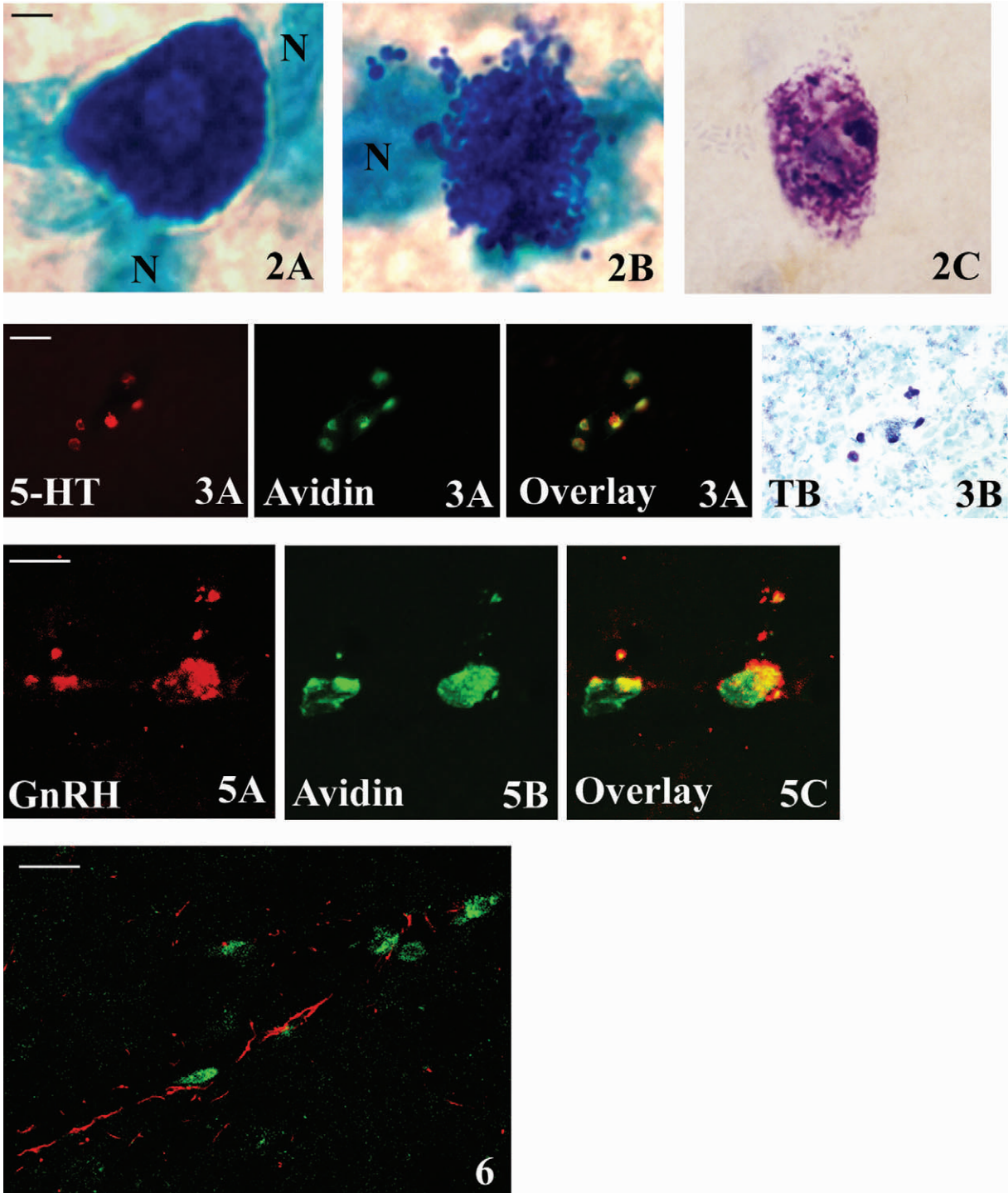
As in mice, prolonged pairing of male rats with estrogen–progesterone primed ovariectomized females resulted in elevation of brain mast cell number in specific thalamic nuclei. Mast cells increased in

Fig. 2. These three rat thalamic mast cells were all stained with acidic toluidine blue which reacts with the sulfated proteoglycans of the granules. In (A) this resting (non-stimulated) cell is uniformly stained and the color is a deep blue/purple. This indicates that the cell is filled with granules. In (B) this activated cell has released granules into the neuropil (arrowheads). In (C) the cell is devoid of mature granules (blue/purple) and contains much paler staining (pale purple/pink) granules. This change in color is due to a loss of the proteoglycans upon exocytosis. The large accumulation of stained material (arrow) is most likely the perinuclear Golgi where packaging of new granules is taking place as the cell recovers. Scale bar = 8 μ m.

Fig. 3. (A) In this section of the thalamus, the mast cells were double-labeled for 5-HT (red) and heparin (revealed by avidin-Cy3 binding; green). The overlay indicates that both products are in granules in each cell. (B) Next the coverslip was removed and the section stained for TB (B). All the mast cells are visualized with the 3 markers used. Scale bar = 30 μ m.

Fig. 5. Double-label immunocytochemistry of brain mast cells in the rat thalamus using antisera directed against mammalian GnRH and 5-HT. Each micrograph is a projection of 7 serially collected 1 μ m optical sections. (A) mast cells with GnRH immunoreactivity (red); (B) mast cells with 5-HT immunoreactivity (green); (C) overlay. Scale bar = 10 μ m.

Fig. 6. In this micrograph the red fluorescence reveals glial astrocytic fibrillary protein (red) in processes associated with a large thalamic blood vessel. These processes are the last cellular element of the blood brain barrier. The cells (arrow) were isolated from rat peritoneal flush and labeled with CellTracker Green and injected intravenously into a host animal. One hour later they are found in the thalamus on the brain side the blood brain barrier. Scale bar = 25 μ m.



Figs. 2, 3, 5 and 6.

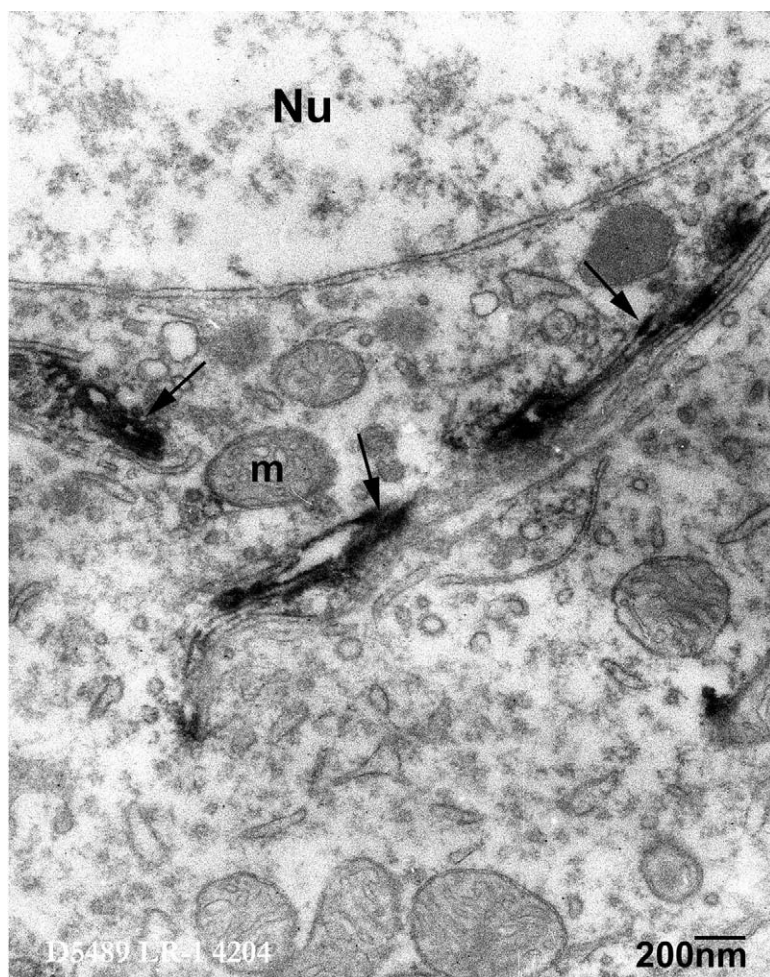


Fig. 4. Electron micrograph of a mast cell in the medial habenula of a 1 mo old dove. The tissue had been processed for ultrastructural demonstration of GnRH immunoreactivity using the LR-1 antibody directed against GnRH. The reaction product was localized to stacks of the Golgi apparatus (arrows). Nu = nucleus; m = mitochondrion. Scale bar = 200 nm.

nuclei of *each* functional thalamic region, i.e., sensory (e.g. VP, Po, dLGN, MGN), motor (e.g. VM), and limbic (e.g. MD, PVT, PT, IAM) areas (Price, 1995). We have found that $28.1 \pm 7.6\%$ of mast cells in unpaired male rats are GnRH-immunoreactive. While the number of mast cells increases with pairing, the proportion of those containing GnRH remains approximately the same (Khalil et al., 2001, and in progress). The increase in the mast cell population in these specific thalamic nuclei may reflect the fact that reproductive pairing involves numerous somatosensory, visual and auditory stimuli, complicated motor responses, and motivational and emo-

tional (i.e. 'limbic') processes. Investigations into the neural control of female copulatory behavior have focused on forebrain pathways (Meisel and Sachs, 1994) and many of the thalamic nuclei are intimately connected with these pathways.

In addition to the mating paradigms noted above, post-partum female rats have more mast cells in their thalami than do age-matched virgin controls. This latter phenomenon is modulated by the complex hormonal changes of pregnancy and parturition or by the stress (see below) of having pups removed for a 4 day period (Silverman et al., 2000).

Brain mast cells and stress

Behavioral manipulations that are independent of reproductive function are also associated with changes in the number of brain mast cells. Immobilization stress (30 minutes) induced intracranial rat mast cell degranulation and elevated mast cell protease levels in the cerebrospinal fluid (Theoharides et al., 1995). This paradigm also increased the permeability of the blood-brain barrier as measured by tracer (^{99}Tc gluceptate) distribution in the brain parenchyma (Esposito et al., 2001). Breakdown in the integrity of the blood-brain barrier induced by brain mast cell activation precedes the onset of clinical symptoms in multiple sclerosis in humans (Goodin et al., 1999) as well as in a rodent model (Kermode et al., 1990). Five day exposure to a novel male rat increased the number of mast cells in the thalamus, perhaps because this pairing was stressful given the potential for aggressive attacks and territorial disputes (Asarian et al., 2002) as has been suggested by experiments in mice (Cirulli et al., 1998).

Mast cells migrate from blood to brain

Cammermeyer (1973) suggested that mast cells may migrate through the area postrema in the dorso-lateral part of the medulla to enter the brain. In the dove, we cannot state whether mast cells migrate from the pia mater or across the endothelium of blood vessels to intraparenchymal locations. We have found that in the dove mast cells are located near the tip of the habenula (where the pia mater attaches to the choroid plexus) after 30–60 minutes of courtship and become more widely distributed within the habenula after 2 hours. The apparent rate of movement of activated mast cells into the medial habenula is consistent with the known rate of movement in vitro (up to 180 $\mu\text{m}/\text{h}$) leading us and others to suggest that increases in mast cell numbers reported under this and other physiological and behavioral conditions may be due to migration of new cells into the brain (*vide supra*). Yang et al. (1999) showed that after pairing for short periods (1–7 days post-coitum), mast cells were found in the velum interpositum (choroid plexus of the third ventricle) while after 15–19 days post-coitum they were abundant in the thalamic parenchyma. This led to the

hypothesis that mast cells are translocated from the velum interpositum on dorsal surface of the brain and become associated with the blood vessels and thalamic parenchyma from days 1 to 19 post-coitum.

We have demonstrated that in rat mature mast cells can enter the normal adult brain via a vascular route. Mast cells labeled with a vital dye and injected intravenously were found in the parenchyma of the thalamus within one hour (Fig. 6, see page 319). The labeled cells represented 10% of the resident mast cells and were found on the brain side of the vascular basal lamina. Note that the rapid rate at which donor mast cells crossed the blood-brain barrier is consistent with the rate of increase in numbers of mast cell in doves, mice and rats following the various behavioral and endocrine stimulations described above (Silverman et al., 2000).

The signaling mechanisms recruiting mast cells to the brain are still obscure (as they are for other immune system cells). The chemoattraction of mast cells has been attributed to chemokines. Numerous mast cell chemoattractants have been described, including MCP-1, RANTES, TGF- β , IL-3, etc. (Bochner and Schleimer, 2001). None, however, have been shown to be specific to the thalamic or pial regions where we and others have reported increases in mast cell number. A chemokine mechanism has been recently described which may account for the selective aggregation of mast cells in the dorsal thalamus. Endothelial cells in blood vessels that supply the dorsal thalamus and choroid plexus express CXCR4 receptors, which bind avidly to the chemokine, stromal cell-derived factor 1 α (SDF-1) (Banisadr et al., 2000). SDF-1 is a CXC chemokine originally isolated from a bone marrow stromal cell line (Tashiro et al., 1993; Nagasawa et al., 1994). In the brain, SDF-1 induces the migration of microglial cells and astrocytes (Tanabe et al., 1997). This ligand is also a potent mast cell chemoattractant. This chemokine facilitates mast cell migration across human umbilical venous endothelial cells in vitro without inducing degranulation (Lin et al., 2000). Only two arteries, the dorsal thalamic artery and the anterior choroidal artery, supply the dorsal thalamus (Scremin, 1995). Thus, a chemokine specialization of the vasculature of the dorsal thalamus might attract mast cells. This subject is under investigation. What other specialized mechanisms regulate their subsequent entry into the

brain parenchyma or otherwise affect their function in these areas are currently unknown.

Another potential mechanism by which mast cells might increase in number is by in situ cell division of resident brain mast cells. Studies using BrDU (a thymidine analogue) in doves, however, found no evidence for mitosis within the mast cell population in the MH, although incorporation was present in neuronal stem cells (unpublished observations from the laboratory).

Mast cells are activated and modulated by reproductive hormones

As mentioned previously, in the ring dove treatment with gonadal steroids results in an increase in the mast cell population in the medial habenula. Pharmacological activation of these cells opens the blood-brain barrier (Zhuang et al., 1996). We therefore tested whether administration of gonadal steroids was accompanied by a change in the activation state of brain mast cells. Electron microscopic analysis revealed that in steroid treated animals a larger percent of their mast cells showed signs of ongoing compound exocytosis or piecemeal degranulation than was seen in control birds treated with cholesterol. Isolated birds and those that had courted for 2 hours had more habenular mast cells in the resting state (Wilhelm et al., 2000). Steroid hormones have been shown to modulate secretion from peritoneal mast cells in vitro. Progesterone triggers selective secretion of 5-hydroxytryptamine and not other mediators (Vliagoftis et al., 1990). Estradiol also augments the release of mast cell mediators in the presence of myelin basic protein (Theoharides et al., 1993), substance P or Compound 48/80 (a polyamine known to stimulate mast cell secretion) (Vliagoftis et al., 1992).

Given these findings it is of interest to determine if mast cells have steroid hormone receptors. Estrogen receptor immunoreactivity is expressed in mast cells located in the human bladder (Pang et al., 1995), human upper airways (Zhao et al., 2001) and rat dura mater (Rozniecki et al., 1999). We have documented using immunocytochemistry that rat thalamic mast cells express estrogen receptor α (unpublished observations from the laboratory). In the upper airways and the bladder of humans, mast

cells, but not lymphocytes, macrophages, or other immune cells, express estrogen and progesterone receptors (Zhao et al., 2001).

Conclusion

Our laboratory has demonstrated for the first time that normal behavior is associated with an increase in the brain parenchyma of mast cells and that they contain immunoreactive GnRH. Furthermore, preliminary results suggest that brain mast cells have the ability to synthesize as well as store GnRH. Mast cells have the ability to migrate rapidly into the brain, to degranulate and to release a plethora of mediators, including GnRH, in the neuropil. Their presence in the brain following/during normal behavioral manipulations could represent a significant neural-immune control mechanism modulating normal brain function in specific CNS regions.

Abbreviations: Thalamic Nuclei

dLGN	dorsal lateral geniculate
IAM	interanteromedial
MD	mediodorsal
MGN	medial geniculate
Po	posterior
PT	paratenial
PVT	paraventricular thalamus
VM	ventromedial
VP	ventroposterior

Acknowledgements

This work was supported by NIH grants MH 54088 (AJS), MH 29380 (RS) and T32 DK 07328-23 (MK).

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